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DEVELOPMENT OF A CHEMICAL METHOD FOR DETECTING PETROGENIC HYDRO--ETC(U)
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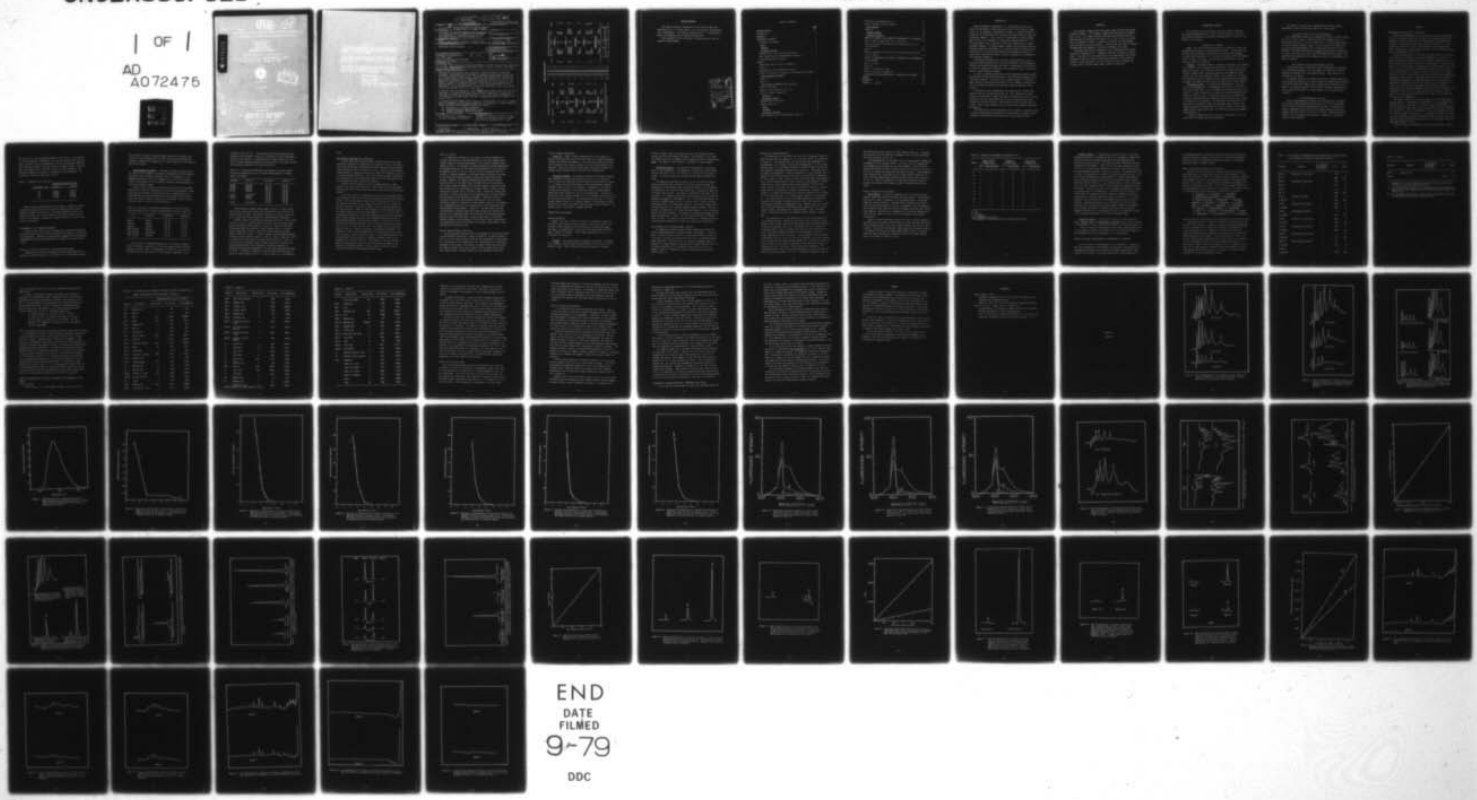

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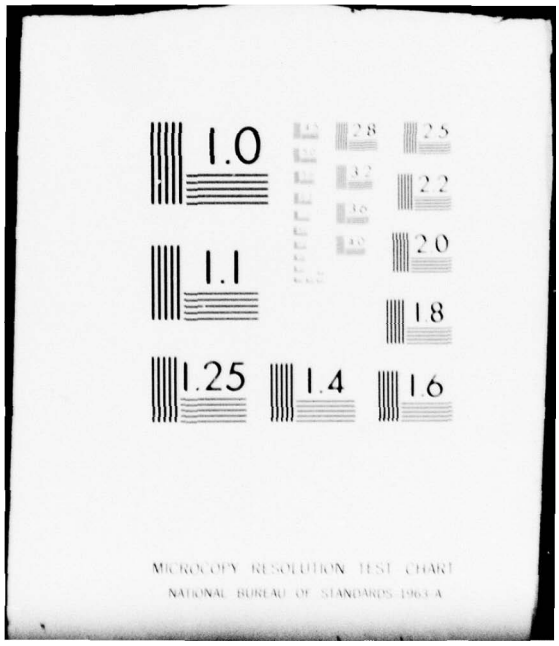
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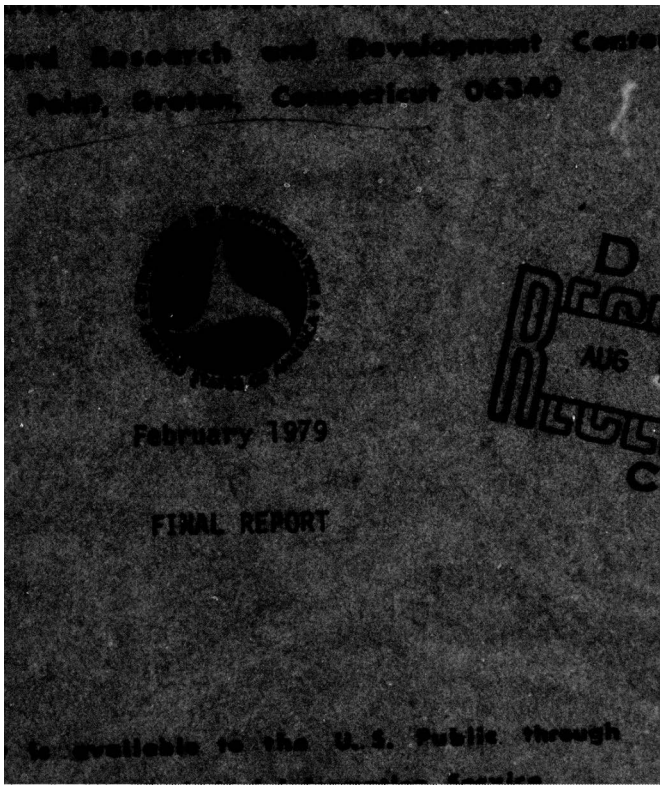
February 1979
FINAL REPORT

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DONALD L. BIRKIMER, Ph.D., P.E.
Technical Director
U.S. Coast Guard Research and Development Center
Avery Point, Groton, Connecticut 06340

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16. Abstract

The purpose of this project was to develop a chemical method for detecting petrogenic hydrocarbons in the presence of biogenic hydrocarbons at levels below those causing biological harm. The method that was developed involves the use of high pressure liquid chromatograph using chloroform as the solvent and measuring fluorescence in the range of 418 nm after excitation at 403 nm. The method is capable of detecting petroleum hydrocarbons in the presence of overwhelming amounts of biological material. It has been demonstrated that the fractions of oil which fluoresce at 418 nm after excitation at 403 nm are among the most persistant fractions in crude oil. Of the six crude oils investigated,

Of the six crude oils employed in this investigation, was Saudi Arabian was the least responsive to the above technique and yet is still detectable in quantities of less than 0.1 µg and easily quantifiable at 1 µg. Under these circumstances oil is detectable in the ppb range using small to moderate sized water samples (one liter or less). microgram microgram,

It has been reported (Brown, 1977) that an oil concentration of 5.5 ppb in water would be harmless to the biological community and, therefore, the newly developed method is capable of detecting oil below toxic levels.

6 **Development of a Chemical Method for Detecting Petrogenic Hydrocarbons in the Presence of Biogenic Hydrocarbons Down to the 5 PPB Level.**

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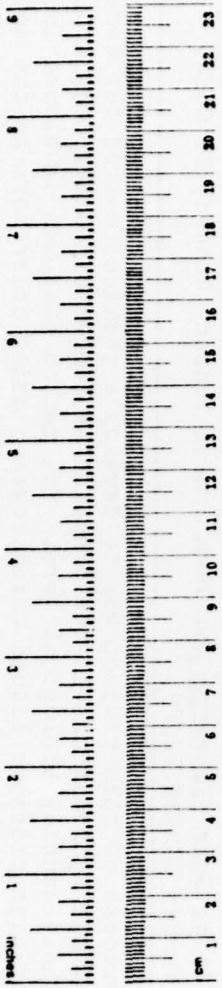
METRIC CONVERSION FACTORS

Approximate Conversions to Metric Measures

Symbol	When You Know	Multiply by	To Find	Symbol
		LENGTH		
in	inches	*2.5	centimeters	cm
ft	feet	30	centimeters	cm
yd	yards	0.9	meters	m
mi	miles	1.6	kilometers	km
		AREA		
in^2	square inches	6.5	square centimeters	cm^2
ft^2	square feet	0.09	square meters	m^2
yd^2	square yards	0.8	square meters	m^2
mi^2	square miles	2.6	square kilometers	km^2
	acres	0.4	hectares	ha
		MASS (weight)		
oz	ounces	28	grams	g
lb	pounds	0.45	kilograms	kg
	short tons (2000 lb)	0.9	tonnes	t
		VOLUME		
tsp	teaspoons	5	milliliters	ml
Tbsp	tablespoons	15	milliliters	ml
fl oz	fluid ounces	30	milliliters	ml
c	cups	0.24	liters	l
pt	pints	0.47	liters	l
qt	quarts	0.96	liters	l
gal	gallons	3.8	liters	l
ft^3	cubic feet	0.03	cubic meters	m^3
yd^3	cubic yards	0.76	cubic meters	m^3
		TEMPERATURE (exact)		
°F	Fahrenheit temperature	5/9 (after subtracting 32)	Celsius temperature	°C

*1 in = 2.54 (exactly). For other exact conversions and more detailed tables, see NBS Misc. Publ. 286, Units of Weights and Measures, Price $2.25, SD Catalog No. C13.10:286.



Approximate Conversions from Metric Measures

Symbol	When You Know	Multiply by	To Find	Symbol
		LENGTH		
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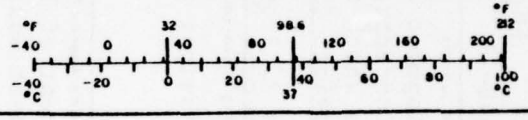
cm	centimeters	0.4	inches	in
m	meters	3.3	feet	ft
m	meters	1.1	yards	yd
km	kilometers	0.6	miles	mi
		AREA		
cm^2	square centimeters	0.16	square inches	in^2
m^2	square meters	1.2	square yards	yd^2
km^2	square kilometers	0.4	square miles	mi^2
ha	hectares (10,000 m^2)	2.5	acres	
		MASS (weight)		
g	grams	0.035	ounces	oz
kg	kilograms	2.2	pounds	lb
t	tonnes (1000 kg)	1.1	short tons	
		VOLUME		
ml	milliliters	0.03	fluid ounces	fl oz
l	liters	2.1	pints	pt
l	liters	1.06	quarts	qt
l	liters	0.26	gallons	gal
m^3	cubic meters	35	cubic feet	ft^3
m^3	cubic meters	1.3	cubic yards	yd^3
		TEMPERATURE (exact)		
°C	Celsius temperature	9/5 (then add 32)	Fahrenheit temperature	°F



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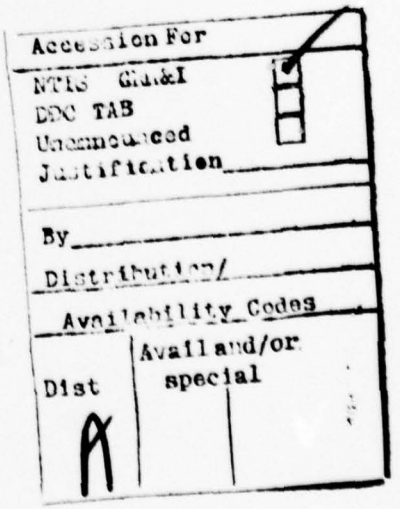

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INTRODUCTION

Despite extensive precautions, it is anticipated that oil will enter the environment at deep-water port sites. In the case of large spills, it is necessary to determine the extent of cleanup required to reduce the oil concentrations to levels below those which will harm the biological community. Also, since it is reasonable to expect chronic small spills, it is essential to ensure that concentrations of oil in the environment do not accumulate to the point where they will cause biological damage. For these reasons, research was initiated which was directed toward the development of a practical method by which the concentration of oil in water and sediments surrounding deep water port sites can be routinely monitored at levels considered to be harmless to the biological community.

Selectivity and sensitivity are the key characteristics of the required methodology-selectivity from the standpoint of distinguishing between petroleum hydrocarbons and biogenic hydrocarbons, and sensitivity from the standpoint of measuring oil concentrations below the levels known to cause biological damage.

In order to address the problem of differentiating between biogenic hydrocarbons and petroleum hydrocarbons, work employing biological materials was conducted. It was felt that a method capable of detecting small amounts of petroleum hydrocarbons in the presence of large quantities of biological materials was the most effective approach to the problem.

A majority of this investigation was conducted with either Saudi Arabian crude oil or Empire Mix crude oil. The rationale for these choices was as follows. Empire Mix crude oil typifies the type of crude produced in the Gulf south area, and of the oils imported into ports situated on the Gulf of Mexico, Saudi Arabian crude oil ranks first in volume. Other crudes imported into the area include Iranian, Venezuelan and Nigerian.

The objective of this investigation was to develop a chemical method of detecting petrogenic hydrocarbons in the presence of biogenic hydrocarbons at levels below those which would cause biological harm.

MATERIALS

All inorganic reagents were ACS reagent grade obtained from Fisher Scientific Company. All inorganic reagents were extracted with hexane prior to use. All organic solvents were pesticide grade distilled in glass obtained from Burdick and Jackson, Inc. Prior to use, all glassware was cleansed in Alconox , bathed in a sulfuric acid--dichromate bath, and baked in an oven overnight. Extreme care was taken to prevent contamination from handling. Only glass containers were used.

The Empire Mix crude oil employed in this investigation was kindly supplied by Standard Oil Co., Pascagoula Refinery, Pascagoula, Mississippi. The other crude oils employed in this investigation were kindly supplied by Exxon Co. from the following refineries: Saudi Arabian, Baytown, Texas; Venezuelan (La Rosa) and Nigerian (LT), Bayonne, New Jersey; and the Iranian (heavy crude), Benicia, California.

EXPERIMENTAL METHODS

The objective of this Task was to develop a method; therefore, only general methodology will be given in this section of the report. The detailed description of the newly developed method is given in the RESULTS of this report.

Preparation of Samples

Water. One liter of water was placed in a separatory funnel and extracted three times with the appropriate solvent. The extracts were filtered through anhydrous sodium sulfate and evaporated in vacuo. The sample was transferred to a vial with ethyl ether which was removed under a stream of nitrogen. The residue was redissolved in the appropriate solvent for analysis.

Sediment. One hundred grams of sediment (wet weight) were weighed into a beaker to which anhydrous sodium sulfate (5-10 gm) and 75 ml of hexane were added. After vigorous stirring, the hexane was decanted through a powder funnel containing glass wool and anhydrous sodium sulfate that had been rinsed with hexane. The contents of the funnel were rinsed with 25 ml of hexane and the combined filtrates evaporated in vacuo. The sample was then treated as described for water samples.

Biological tissue. All tissues were homogenized in distilled water as 50% homogenates. The homogenate was placed in a round bottom boiling flask with 5 ml of 0.75 MKOH in 2% aqueous MeOH per gram of tissue. A carbon boiling chip was added and the sample refluxed overnight and allowed to cool. Twenty-five milliliters of hexane were carefully poured through the condenser into the boiling flask to wash the interior of the condenser. The mixtures in the flask were then poured into a separatory funnel and the hexane layer removed. The sample was extracted three times with 25 ml of hexane. The combined hexane extracts were washed with 10 ml MeOH and evaporated in vacuo to approximately 2 ml.

"Spiked" samples were those to which a known amount of oil was added after the sample had been homogenized.

"Oil-exposed" organisms were organisms obtained from bioassay experiments in which oil had been added to the test system.

Open-End Silica Gel Column Chromatography

The concentrated extract of the sample was placed on a glass column (20 cm x 1.0 cm ID) with a 70-100-n-Fritted disc packed with 4.5 g 60-200 mesh activity I silica gel topped with 2.5 g 80-200 mesh activity I neutral aluminia packed in hexane. The concentrate was washed onto the column with approximately 5 ml of hexane and eluted with 100 ml benzene. The benzene was evaporated invacuo and the sample transferred to a small vial with ethyl ether which was removed under nitrogen. The sample was then redissolved in a measured volume of the appropriate solvent for analysis.

High Pressure Liquid Chromatographic Analyses

A Waters Associates ALC/GPC-502 liquid chromatograph was employed for UV absorption analyses. The instrument was fitted with a FS-770 Schoeffel fluorometer for fluroescence studies. Chart speed for all analyses was 0.5 cm/min.

All samples injected into the HPLC utilizing a methanol-water solvent system were dissolved in a known quantity of hexane before injection. All other solvents were removed under nitrogen before addition of the hexane. All samples injected into the HPLC utilizing a chloroform solvent system were dissolved in a known quantity of chloroform before injection.

Gas Chromatographic Analyses

The gas chromatographic analyses were conducted using a Beckman GC-45 with a flame ionization detector and a 6' x 1/8" OD stainless steel 3% SE-30 on 80-100 mesh Chromasorb W column. The injector and detector temperatures were 300C and the column temperature was programmed from 100 to 300C at a rate of 3^{o} per min. Qualitative identification of the components was achieved by comparison of retention times to known standards.

RESULTS

PRELIMINARY RESULTS USING UV ABSORPTION

Previous investigations in our laboratory had shown that quantification of ultraviolet light (UV) absorption by selected aromatic constituents of oil was satisfactory for estimating the concentration of crude oil in biological tissues under certain conditions, (1). The technique involved the use of a high pressure liquid chromatograph (HPLC) containing a fixed wavelength detector (254 nm) using a methanol-water solvent system (70:30, v:v).

With the acquisition of a variable wavelength detector experiments were conducted to determine the optimum wavelength for operation. A series of tests was performed at 10 nm increments, between 224 and 334 nm with Empire Mix crude oil and with ten gram samples of oyster tissue. Figure 1 is a representative chromatogram obtained from Empire Mix crude oil, while Figure 2 displays representative results obtained using the oyster tissue samples. Particular attention was given to the region between 254 and 334 nm since only polycyclic aromatic hydrocarbons show absorbance in this region. Wavelengths below 254 nm are not desirable because many compounds, including unsaturated fatty acids from the organisms will absorb UV light in this region as was confirmed by this study. Also many possible contaminants from solvents, glassware, fingerprints, and other environmental sources (e.g. food consumed by the organisms) are more likely to show significant absorptions in this region. Solvents such as benzene will also absorb in this region and thus obscure oil components.

The region between 274 and 284 nm seemed to be most appropriate for several reasons. A wavelength of approximately 274 nm maximized the peaks from the oil components at retention times of approximately 4.50 and 6.00 minutes (these peaks are not obtained from biological samples) and reduced by a factor of approximately three the background from the 10 g oyster samples (see Figure 2). A wavelength of greater than 300 nm would have greatly reduced the background from the oyster, but would have also greatly reduced the sensitivity of the instrument toward oil components (Figures 1 and 2).

The next series of experiments determined the response of Empire

Mix crude oil at each wavelength between 274 and 284 nm. On the basis of these experiments a wavelength of 277 nm was selected since the peak with a retention time of approximately 6.00 min was maximized (Table I). This peak was not found in mullet, shrimp, or oyster tissues. The peak with a retention time of 4.50 min, which also did not appear in the biological samples, was almost at a maximum at this wavelength. The deviation in retention time was $\pm$ 0.25 min.

Table I. Maximization of Peak Area

	Relative Peak Area (Counts)	
wavelengths (nm)	6.00 min	4.50 min
274	1965495	1296702
276	2588259	1404661
277	2596622	1402633
278	2437360	1412309

Figure 3 shows representative results using 45 samples of control organisms (shrimp, mullet, and oyster) and organisms that had been spiked with Empire Mix crude oil. These data demonstrate that it is possible to detect oil in the presence of biological material on the basis of the presence of peaks with retention times of 4.50 $\pm$ 0.25 and 6.00 $\pm$ 0.25 minutes.

DEVELOPMENT OF THE FLUORESCENCE METHOD

From this early work it did not appear that UV absorption measurements would yield the selectivity and sensitivity demanded of the method being developed. Theoretical considerations suggested that fluorescence measurements would increase sensitivity and might also increase selectivity.

Effect of Variation of Excitation and Emission Wavelengths

Simultaneous studies were performed with the Aminco-Bowman Fluorometer and the HPLC coupled to a Schoeffel FS 970 fluorescence detector.

The rationale was that the Aminco-Bowman system would determine properties of total samples while the other system would determine properties of samples after separation on a HPLC column with a 70% MeOH-30% H_2O solvent system.

Aminco-Bowman Fluorometer. Fractions of shrimp, oysters, mullet, and crude oils were tested to determine optimum excitation and emission wavelengths and to establish minimum levels of detectability. The maximum detectability of all oil samples was at a 10^6-fold dilution (Table II and Figures 4-10) for samples previously chromatographed on a silica gel open-end column.

Table II and III indicate the results obtained upon fluorescence examination of selected fractions from crude oils and organisms. These fractions were obtained by placing either oil or hexane extracts of organisms upon an open-end silica gel column and then eluting successively with hexane, benzene, chloroform, and methanol (100 ml of each solvent/0.1 gm of crude oil or tissue extract).

Table II. Excitation and Emission Peak Maxima for Various Obtained from Open-End Silica Gel Column Chromatography of Several Crude Oils

Oil	Fraction	Excitation	Emission
Empire	Hexane	300 nm	418 nm
Iranian	Hexane	303 nm	357 nm
Nigerian	Benzene	328 nm	375 nm
Saudi Arabian	Benzene	403 nm	435 nm
Saudi Arabian	Chloroform	432 nm	472 nm
Saudi Arabian	Methanol	425 nm	464 nm

In all cases, the minimum detectability for biological samples (Table III) was at a dilution of 10^3-fold on samples from an open-end silica gel column. This is considerably less than for crude oil and indicates that the fluorescence of the biological organisms is low

compared to that for oil. The maximum excitation and emission wavelengths of the biological fraction are uniformly of lower wavelengths than the oil fractions. It appeared that the best monitors for oil would be those compounds having excitation maxima in the region 300-400 nm and emission maxima in the region 400-500 nm.

Table III. Excitation and Emission peak maxima for Various Eluates Obtained from Open-End Silica Gel Column Chromatography of Shrimp, mullet, and Oyster

Organism	Fraction	Excitation	Emission
Shrimp	Benzene	234 nm	350 nm
Shrimp	Benzene	234 nm	354 nm
Mullet	Benzene	303 nm	355 nm
Mullet	Benzene	290 nm	350 nm
		235 nm	350 nm
Mullet	Chloroform	292 nm	350 nm
Oyster	Benzene	238 nm	355 nm
Oyster	Hexane	235 nm	350 nm
		290 nm	350 nm
Oyster	Chloroform	292 nm	350 nm

Three different samples (Saudi Arabian crude oil, tissue spiked with the oil, and tissue alone) were fractionated on an open-end alumina-silica gel column. The benzene fractions of shrimp, oyster, and mullet tissue were then observed for long wavelength fluorescence.

Figure 11 shows the fluorescence emission spectrum of Saudi Arabian crude oil (curve 1), shrimp tissue only (curve 2), and shrimp tissue spiked with Saudi Arabian crude oil (curve 3). Figure 12 shows the fluorescence emission spectrum of Saudi Arabian crude oil (curve 1), oyster tissue only (curve 2), and oyster tissue spiked with Saudi Arabian crude oil (curve 3). Figure 13 shows the fluorescence emission spectrum of Saudi Arabian crude oil (curve 1), mullet tissue only curve 2), and mullet tissue spiked with Saudi Arabian crude oil (curve 3). From these data it appeared that it might be possible to detect compounds from petroleum in the presence of biological material by employing an excitation wavelength of 403 nm and an emission wavelength of 450 nm. It should be pointed out that the sharp emission peak at 400 nm in these Figures (11-13) is a scatter peak, not a true emission

peak.

HPLC Using a Schoeffel FS 770 Detector

Initial tests were conducted under the same conditions as those previously developed for maximization of selectivity with a UV detector. The solvent system was 70% methanol-30% water and the excitation wavelength setting on the UV detector was 277 nm. Figure 14 shows typical chromatograms for identical quantities of Empire Mix crude oil using the UV and fluorescence detectors. Thus, even without optimum conditions, the sensitivity of the fluorescence detector is four times greater than that achieved with the UV absorption detector.

Analyses were performed on oyster specimens exposed to small amounts of oil and it was found that in many cases, oil was not demonstrable using the UV detector but was easily seen using the fluorescence detector.

In the next series of experiments the excitation wavelengths were varied between 230 nm and 450 nm and the emission measured using various filters. The filters employed were the KV 370 (range 340-370 nm), KV 389 (range 370-405 nm), KV 418 (range 400-420 nm), KV 470 (range 415-505 nm), and the KV 550 (range 515-600). Both oil and biological samples were analyzed. Figure 15 shows samples of Saudi Arabian crude oil and oyster tissue at excitation wavelengths of 245, 275, and 403 nm. An operational wavelength of 274 nm was selected for use with the methanol - water solvent system since this value maximized emission from the oil and minimized emission from biological samples. Verification of this method was performed by spiking a series of mullet, shrimp, and oysters with 12.5, 25, 50, 100, and 200 ug/g of the benzene eluate from Saudi Arabian crude oil. Figure 16 presents a representative example. The presence of crude oil was demonstrable at a concentration as low as 12.5 ug/g in mullet, shrimp, and oyster tissue. Oil was demonstrated in the spiked samples by the presence of peaks with retention times of approximately 11.5 and 16.5 minutes. The amount of crude oil was determined by integrating the areas of these two peaks and comparing the results with a known standard. Figure 17 shows a calibration curve obtained utilizing the conditions described above.

Effect of Solvent

As indicated previously in this report, the Aminco-Bowman fluorometer demonstrated a maximum selectivity of oil over biogenic hydrocarbons using an excitation wavelength of 403 nm and an emission wavelength of 450 nm. In contrast, the HPLC-fluorescence system demonstrated the maximum selectivity at an excitation wavelength of 274 nm and with an emission filter of 370 nm. Tables II and III indicated that the majority of samples examined by the Aminco-Bowman fluorometer had been in the relatively nonpolar hexane, benzene, and chloroform solvent systems. However, the samples examined with the HPLC-fluorescence system had been in the highly polar 70% MeOH-30% H_2O solvent system. The solvents hexane, benzene, and chloroform cannot be utilized in the HPLC system with the μ Bondapack C_{18}/corasil column. However, chloroform may be utilized with a μ Porasil column. Therefore, studies were performed to compare chloroform to the methanol-water solvent system at varying excitation and emission wavelengths. Figure 18 vividly illustrates that the 403 nm excitation setting with a KV-418 filter (400-420 nm range) is sensitive to Saudi Arabian crude oil. Furthermore, the chromatogram shows only one sharp peak at a short retention time (approximately three minutes). Figure 19 shows data under identical circumstances for shrimp tissue and it may be seen that there were no peaks on the chromatogram using an excitation of 403 nm and measuring emission using a KV-418 filter. Since an excitation of 403 nm and emission measured at approximately 418 nm (KV-418 filter) demonstrates the maximum selectivity of petroleum over biogenic hydrocarbons, these settings were selected for use.

Results Using Different Crude Oils

Using the method described above, the response of the benzene eluates of five different crude oils is shown in Figure 20. It may be observed that all of the crudes yielded a peak and the differences in peak heights are a reflection of the variations in the concentrations of fluorescing compounds in the crudes. The results in Figure 20 were obtained using the benzene eluates (silica gel column) of 100 μg quantities of Nigerian, Saudi Arabian, Iranian, Empire Mix, and Venezuelan crude oils.

Effect of Sample Preparation

Crude Oil. Figure 21 shows representative 100 µg samples of several oils which were eluted from the silica gel column with hexane, benzene, chloroform, and methanol. The hexane and methanol fractions were eliminated from further consideration since they showed no fluorescence. While the chloroform eluate gave the greater response, the benzene eluate provided results that were more reproducible.

Biological Samples. The results obtained with biological samples were comparable to those described above for the oils in that the benzene eluate gave the most reliable results as illustrated in Figure 22. As shown, the chloroform eluate of the representative organism (shrimp) failed to give a flat baseline. Consequently, the results obtained upon injection of the chloroform eluate of shrimp samples spiked with Saudi Arabian oil are not as reproducible. The error in injections of the sample averaged 3.7% for the benzene fraction and 6.7% for the chloroform fraction. The chloroform fraction had a 7.1% error between samples versus a 3.4% between samples of the benzene fraction. Therefore, all results described from this point forward pertain to the benzene eluate of the specific samples involved.

DESCRIPTION OF THE METHOD

Sample Preparation

Water. One liter of water is placed in a separatory funnel and extracted three times with 50 ml of hexane. The hexane extracts are filtered through anhydrous sodium sulfate and evaporated *in vacuo*. The sample is transferred to a vial with ethyl ether which is removed under a stream of nitrogen. This is then redissolved in 10 µl of chloroform for injection.

Sediment. One hundred grams of sediment (wet weight) is weighed into a beaker to which anhydrous sodium sulfate (5-10 gm) and 75 ml of hexane is added. After vigorous stirring, the hexane is decanted

through a powder funnel containing glass wool and anhydrous sodium sulfate that has been rinsed with hexane. The contents of the funnel are rinsed with 25 ml of hexane and the combined filtrates evaporated *in vacuo*. The sample is then treated as described for water samples.

Biological Material. All tissues are homogenized in distilled water as 50% homogenates. The homogenate is placed in a round bottom boiling flask with 5 ml of 0.75 M KOH in 2% aqueous MeOH per gram of tissue. A carbon boiling chip is added and the sample refluxed overnight.

The samples are removed and allowed to cool for several minutes. Twenty-five milliliters of hexane are carefully poured through the condenser into the boiling flask to wash the interior of the condenser. The mixtures in the flask are then poured into a separatory funnel and the hexane layer removed. The sample is extracted three times with 25 ml hexane. The combined hexane extracts are washed with 10 ml MeOH and evaporated *in vacuo* to approximately 2 ml. The concentrated extract is placed on a glass column (20 cm x 1.0 cm ID) with a 70-100-μ-Fritted disc packed with 4.5 g 60-200 mesh activity I silica gel topped with 2.5 g 80-200 mesh activity I neutral aluminia packed in hexane. The concentrate is washed onto the column with approximately 5 ml of hexane and eluted with 100 ml benzene. The benzene is evaporated *in vacuo* and the sample transferred to a small vial with ethyl ether which is removed under nitrogen. The sample is then redissolved in a measured volume of chloroform and injected into the HPLC for analysis by fluroescence.

High Pressure Liquid Chromatographic Analysis

All samples are analyzed using a Waters Associates ALC/GPC-502 liquid chromatograph with a FS-970 Schoeffel fluorometer. A 30 cm x 6 mm OD μ Porasil column is used with a chloroform solvent flow rate of 1.0 ml/min. The data are analyzed with the help of a Hewlett-packard 3380A integrator. The area of the oil peak in an unknown sample is compared to a graph prepared from injection of known amounts of oil. In all cases a graph has to be prepared for each kind of oil. As an example, Figure 23 illustrates a typical graph prepared with Saudi Arabian crude oil.

Sensitivity and Reproducibility

The sensitivity of the method was verified by injecting a series of Saudi Arabian crude oil samples (1 µg, 10 µg, and 100 µg) as illustrated in Figure 24. The minimum level of detectability was determined to be approximately 1 µg. Figure 25 shows the chromatograms of 1 µg of Saudi Arabian crude oil at the same attenuation as shown in Figure 24 and the most sensitive instrument setting that is practical. This means that if one makes the assumption of 100% recoverability of oil from water that a samples of only one milliliter would have to be collected to determine oil at a level of 1 ppm. Figure 26 shows the effect when varying quantities of Saudi Arabian crude oil were placed in 1 gram samples of shrimp versus the oil alone. Similar results were obtained for mullet and oyster tissues. These data show that this method of analysis is quantitative even in the presence of biogenic hydrocarbons. However, the presence of biogenic hydrocarbons does lower the sensitivity by a factor of five (from less than 1 µg of Saudi Arabian crude oil to less than 5 µg). The reason for this is that the sample preparation is much more extensive for biological tissues, and as a result the recovery of petroleum hydrocarbons is approximately 20%. However, even in the worst case from an environmental standpoint (Saudi Arabian crude oil in biological tissue), the sensitivity is approximately 5 ppm. The sensitivity of the method in water and sediments varies between 1 and 100 ppb.

The reproducibility of the method for oil alone was determined in the following fashion. Repetitive injections (Ten) of 100 µg of Saudi Arabian crude oil were analyzed and the percent error for these repetitive injections calculated to be 1.3%. The percent error for repetitive injections (Ten) of 100 µg of Empire Mix crude oil was calculated to be 3.4%. One gram of Empire Mix crude oil was dissolved in 100 ml of hexane producing a 10 µg/µl solution. Ten injections of five different concentrations (20 µg, 40 µg, 60 µg, 80 µg, and 100 µg) of this solution were injected and the resulting data graphed into a standard calibration curve of area in unit counts as produced by the integrator versus concentration in µg. The slope of the line was then calculated. The standard deviation from the slope was determined to be 0.62 and the percent error was calculated to be 6.1%. A standard calibration curve as

described above was calculated for Saudi Arabian crude oil. The standard deviation from the slope was determined to be 0.15 and the percent error was calculated to be 2.5%

The reproducibility of the method for oil in biological tissues was determined in the following fashion. A total of 100 g of shrimp meat was homogenized and divided into ten equal samples. Each of the ten samples was spiked with Saudi Arabian crude oil at the rate of 100 µg of oil per g of shrimp and treated as described previously. Ten subsamples (each theoretically containing 100 µg of Saudi Arabian crude oil) of each of the ten samples were analyzed and the percent error calculated to be 3.7%. This is considered to be excellent reproducibility for residue type samples which undergo a number of manipulations during workup.

Results Obtained Using the Method

Water Samples. In previous examinations of one liter sea water samples, extraction had been performed only with hexane. Chloroform was utilized in a study to determine if an increased yield of fluorescent compounds could be achieved. (Benzene forms an emulsion with water.) While chloroform recovered 74% of the oil compared to 55% for hexane, it also extracted three times the amount of biogenic materials from control water samples. Therefore, all subsequent one liter water samples were extracted with hexane in order to avoid interference from biogenic hydrocarbons.

Figure 27 shows representative data for tests of 100 ml samples of sea water taken from tanks containing mullet versus sea water taken from tanks containing mullet, and 1 mg of Saudi Arabian crude oil per liter of water. The control tank showed a small peak with a retention time of 2.07 minutes, while the oil-treated tanks showed a peak (3.12 minute retention time) area which is equivalent to 100 µg of Saudi Arabian crude oil in 100 ml of water or 1 mg/l.

Table IV. Analyses of sediments from ponds for oil

Sample	Saudi Arabian Treated Pond		Nigerian Treated Pond		Empire Mix Treated Pond	
	UV*	Fluorescence**	UV	Fluorescence	UV	Fluorescence
N	+	+	0	0	0	+
N_1	+	+	0	0	0	+
S	+	-	0	0	0	-
S_1	+	+	0	0	+	+
E	+	+	0	0	+	0
E_1	+	+	+	+	0	+
W	0	0	+	0	0	+
W_1	+	+	0	0	0	+
C	+	0	0	0	0	0
C_1	+	+	0	0	0	0

+ = oil
0 = no oil
- = no sample
* UV = adsorption at 274 nm
** Fluorescence = excitation at 403 nm, emission at 418 nm

Sediment Samples. To demonstrate the applicability of the newly developed technique for estimating crude oil in sediments, samples were collected from four estuarine ponds which had been employed in an earlier oil pollution study. The ponds contained a naturally developed organically rich sediment. Three of the ponds were treated with crude oil (22.6 l of oil per pond containing 5 million liters of salt water). One pond remained untreated to serve as a control. Ten months after the oil spill, ten samples were collected from each pond and analyzed. Figure 28 shows representative chromatograms for a sample taken from the control pond and a sample taken from the pond which had been treated with Saudi Arabian crude oil. The striking feature is that the presence of oil is still clearly indicated in the treated pond sample even after ten months. The tiny blip in the control sample was apparently a result of a minute amount of contamination. A comparison of the results obtained using UV adsorption and fluorescence on sediments containing large amounts of biogenic materials is given in Table IV. It is interesting to note that there was a discrepancy between the two types of analyses. Samples indicating oil by UV adsorption but not by fluorescence were a reflection of the high organic content of the samples and illustrate the impact of biogenic hydrocarbons on the method. Samples indicating oil by fluorescence but not by UV adsorption attest to the increased sensitivity of the newly developed method.

Biological Samples. The uptake of crude oil by two species of algae (*Carteria chuii* and *Chaetoceros curvisetus*) after 96-hrs of growth in the presence of 4 mg/l Saudi Arabian crude oil is shown in Figure 29. There is clearly no interference from biogenic hydrocarbons in the control samples; however, the presence of oil is definitely evident in the organisms which had been exposed to oil during growth.

EFFECT OF BIOGENIC HYDROCARBONS ON PERFORMANCE OF THE METHOD

One of the major considerations in the development of a method to meet the requirements of the "Request for Proposal," was the ability to distinguish petrogenic hydrocarbons from biogenic hydrocarbons. Some results on this phase of the investigation have already been reported

in preceding sections of this report and this section will give more extensive results in respect to the ability of the newly developed oil detection method to discriminate between hydrocarbons of petrogenic origin and those of biogenic origin.

Tests of Marine Algae Grown in the Laboratory

Algae are one of the groups of organisms that could contribute large quantities of biogenic hydrocarbons to the environment. Therefore, pure cultures of twenty-four different marine algae were obtained from commercial sources. The organisms were grown in the appropriate medium. After growth, the organisms were harvested, and prepared for analyses as previously described. None of the organisms listed below contained compounds that fluoresce at a wavelength of 418 nm when excited at a wavelength of 403 nm when using chloroform as the solvent.

(1) Cladophora, (2) Trailliella, (3) Ectocarpus, (4) Enteromorpha intestinalis, (5) Sphacelaria (6) Dictyota ichotoma, (7) Polysiphona, (8) Percusaria percursa, (9) Seirospora, (10) Derbesia, (11) Acrochaetium, (12) Bryopsis, (13) Rhodymenia, (14) Phodochotron, (15) Valonia, (16) Spermothamnion, (17) Agardhiella, (18) Ulua, (19) Cyclotella, (20) Porphyridium, (21) Bangia (22) Petrocladia, (23) Callithamnion, and (24) Polysiphonia

Tests of Estuarine Phytoplankton and Zooplankton Grown in the Laboratory

During the course of a previous investigation concerned with the effects of oil pollution, a number of species of estuarine phytoplankton and zooplankton were isolated and cultured in the presence of oil and in the absence of oil. Samples of the above which had been archived were retrieved and subjected to analyses by the oil analysis method described in this report. As may be observed in Table V oil was only found in those samples which had been grown in the presence of oil. Furthermore, none of the control samples indicated oil by this method. Many of the control samples (no oil) indicated the presence of oil when analyzed by another fluorescence method and one of the samples which had been grown in oil failed to indicate oil when analyzed by the gas chromatographic method.

Table V. Oil content of phytoplankton and zooplankton after growth either in the presence or absence of Empire Mix crude oil

Exposure	Organism	Oil detected* by method in this report	LC**	GC***
Control	Thalassiosira decipiens	-	796	0
EMC oil	"	+	7684	1917
Control	Lithodesmium undelatum	-	952	0
EMC oil	"	+	1718	15
Control	"	-	87	0
EMC oil	"	+	4568	3607
Control	Isochrysis galbana	-	1693	0
4 ppm EMC	"	+	21904	2960
Control	Chactoceros curvisetus	-	2305	0
4 ppm EMC	"	+	4338	59
Control	Skeletonema coastatum	-	0	0
4 ppm EMC	"	+	9210	503
Control	Thalassiosira decipiens	-	1600	0
4 ppm EMC	"	+	9735	269
Control	Chactoceros curvisetus	-	1037	0
4 ppm EMC	"	+	4552	163
Control	Thalassiosira decipiens	-	0	0
4 ppm EMC	"	+	7271	900
Control	Chactoceros curvisetus	-	727	0
4 ppm EMC	"	+	1212	0
Control	"	-	0	0
4 ppm EMC	"	+	1222	195

Table V. (Cont'd)

Exposure	Organism	Oil detected* by method in this report	LC**	GC***
Control	Carteria chuli	-	0	0
EMC oil	"	+	1529	14

* Presence of oil using fluorescence with chloroform as the solvent, excitation at 403 nm, emission at 418 nm.

** Oil calculated on the basis of the quantity of n C-16 in Empire Mix crude oil using gas chromatography as described by Miles, Coign, and Brown (1975).

*** Oil calculated on the basis of total counts (fluorescence at 274 nm) as described by Miles, Coign, and Brown (1975).

Tests of Specimens from Field Collections Obtained from the Mississippi Sound

A variety of different biological specimens were obtained by trawling in the Mississippi Sound. Immediately after collection the specimens were placed in individual containers and cooled in an ice chest. One gram samples of the specimens were prepared and analyzed as previously described. None of the samples contained materials which fluoresce at a wavelength of 418 nm using chloroform as the solvent when excited at 403 nm. Organisms tested were:

(1) dusty flounder, (2) cutlass fish, (3) pinfish, (4) sand sea fish, (5) mullet, (6) shrimp, (7) oyster, (8) blue crab, (9) jelly fish, (10) croaker, (11) sea catfish, (12) menhaden, (13) spade fish, (14) hog choker, and (15) midshipman.

Tests of Specimens from Field Collections Obtained from the Gulf of Mexico

The opportunity arose to acquire biological specimens from the Gulf of Mexico through the efforts of Mr. Melvin Light of the U.S. Coast Guard R & D Center. During several cruises of the ACUSHNET, a wide variety of environmental samples were collected from the vicinities of the three proposed deep water port sites. The samples were appropriately packaged and frozen immediately. The samples were analyzed by three separate methods: (1) measuring fluorescence at 418 nm after excitation at 403 nm using chloroform as the solvent, (2) measuring fluorescence at 370 nm after excitation at 274 nm using chloroform as the solvent, and (3) measuring fluorescence at 370 nm after excitation at 274 nm using methanol: water as the solvent system. The results of these analyses are given in Table VI. It is interesting to observe that 67% of the samples did not show oil using fluorescence measured at 418 nm after excitation at 403 nm with chloroform as the solvent system while both of the other methods indicated oil in 100% of the samples.

EFFECT OF WEATHERING AND MICROBIAL DEGRADATION ON PERFORMANCE OF THE METHOD

Effect of Aging

The fluorescence of a fresh sample of Empire Mix Crude Oil was

Table VI. Oil content of environmental samples collected from the Gulf of Mexico using three different methods of analyses

Station	Description	Determinations in ng oil/g wet wt		
		403 nm $CHCl_3$	274 nm $CHCl_3$	274 nm $MeOH/H_2O$
A2/1	Neuston tow	0	359	5139
A2/2	Grab #1	1	69	629
A2/3	worm	2292	24589	598861
A2/4	Grabs	1329	17237	117977
A2/5	Neuston tow	0	1945	7450
A2/6	Grab #1	2	706	2832
A2/7	sand dollar	0	1790	7927
A2/7	snail & worms	2416	12963	104157
A2/9	starfish	12	15012	203286
A2/10	starfish & muscle	1041	17333	434639
A2/11	Grabs	99	1291	13411
A2/12	sea horses	0	256	3335
A2/12	starfish & shrimp	1942	14209	159193
A2/13	Neuston tow	0	319	2162
A2/14	from Sargasum	529	5383	55136
A3	Neuston tow	0	696	5196
AB	Neuston tow	595	7199	44219
B4/AB	yellow fin liver	697	8660	53459
B4/AB	yellow fin meat	0	4205	64259
B5	squid	0	565	3668
B6/5	Neuston tow	3201	19724	238990
B6/6	sea catfish liver	0	8331	122516

Table VI. (Cont'd)

Station	Description	403 nm $CHCl_3$	274 nm $CHCl_3$	274 nm $MeOH/H_2O$
B6/6	sea catfish muscle	0	4085	54938
B6/11	Neuston tow	0	1652	63293
B6/11	croaker liver	0	20224	352998
B6/11	croaker muscle	0	4083	81255
B6/14	Neuston tow	0	257	8648
B6/15	Plankton tow	0	175	3870
B6/19	hard head catfish liver	0	12567	100120
B6/19	hard head catfish muscle	0	4015	60392
B6/20	gaffsail catfish liver	0	13945	98673
B6/20	gaffsail catfish muscle	0	2246	63035
C1	tuna liver	0	6142	125013
C1	tuna meat	60	4513	101722
C1	tuna liver	0	4559	55776
C1	tuna meat	0	3469	59190
C1	pilot fish	1023	11872	53649
C2	Neuston tow	0	460	13623
D6/1	Grab #2	2855	45534	472874
E1	fish liver	139	28636	55066
E1	fish meat	0	5902	34935
F8	Neuston tow	0	370	7765
G1	Neuston tow	0	513	132949
	flying fish gut found on deck 2/3 of way between G1 and H5	27	9434	82733

Table VI. (Cont'd)

Station	Description	403 nm $CHCl_3$	274 nm $CHCl_3$	274 nm $MeOH/H_2O$
	flying fish meat	101	4987	26118
H6/2	lined sole	0	3875	10803
H6/2	crab	0	553	17742
H6/4	plankton tow	695	43363	349003
H6/5-5	fish	29	6882	55248
H6/8	Neuston tow	0	297	1101
H6/8-4	Neuston tow	1165093	6186	28678
H6/9	Neuston tow	0	404	14564
H6/11	flying fish	0	627	12551
H6/13-3	worm from sed. H5/4	0	5801	68581
H6/14	sea horses	0	4939	58428
H6/14	crab	0	733	9585
H6/20	fish liver	0	12077	521332
H6/20	fish muscle	0	5541	83306
H7	gaffsail catfish liver	0	4963	69463
H7	gaffsail catfish muscle	0	3647	62063
H7/5	tubeworm	0	3758	77129
	shark (4') liver	0	13445	90863
	shark (4') muscle	0	947	22206
	shark (1') liver	0	19085	70754
	shark (1') muscle	0	4714	76060
	squid	0	2949	98506
	crabs	44	4548	31029

compared to the fluorescence obtained from a sample which had been stored in the laboratory for five years. The data in Figure 30 indicates a decrease in fluorescence of 26-35% during storage for five years.

In another experiment, a series of marine sediment samples were incubated with a very low level (23-230 ppb) of oil for a period of up to 60 days under aerobic conditions. The aliphatics had disappeared within 20 days while compounds which emit at 418 nm after excitation at 403 nm were still present after 20 days but were absent after 60 days. This indicates that even though the compounds being detected by the newly developed method are persistant, they do undergo degradation.

During the course of a previous investigation crude oil contained in one gallon plastic jugs were sunk in estuarine test ponds. It was decided to retrieve the jugs and some of the residual oil was collected and subjected to analyses. The samples of Nigerian crude did contain aliphatics and showed oil using HPLC and fluorescence at 274 nm as well as by fluorescence using 403 nm excitation and 418 nm emission. No aliphatic hydrocarbons were observed in samples of the Saudi Arabian crude as determined by gas chromatographic analyses but oil was evident when analyzed by HPLC using 403 nm excitation and 418 nm emission. The material had to be concentrated five-fold in order to demonstrate the presence of oil using HPLC and measuring fluorescence at 274 nm. These data help substantiate that the compounds in oil that emit at 418 nm after excitation at 403 nm are among the most persistent compounds in crude oil as shown by the analyses of the Saudi Arabian crude after remaining in the environment for three years.

Effect of Ultraviolet Light

One of the factors which could have an effect on oil in the environment is ultraviolet irradiation. Consequently, Empire Mix crude oil was subjected to UV irradiation for varying lengths of time. For this experiment, 5 ml of oil was placed in a petri dish bottom, the dish covered with Saran Wrap, and irradiation carried out using a Blak-ray Model B-100A, (Ultraviolet Products, Inc.) at a distance of 16 cm from the sample. After irradiation, three drops of oil were taken

from each sample and diluted to 2 ml with the samples and were analyzed for oil by measuring fluorescence at 418 nm after excitation at 403 nm using chloroform as the solvent. No evidence of change was detected and thus, it appears that UV irradiation will have little or no effect on the oil detection method developed in this investigation since the irradiation was greater than that which would be expected to occur in nature.

Effect of Microbial Degradation

It is known that oil in the environment will weather. This is a term that covers a multitude of events including volatilization and microbial degradation of oil components. Therefore, a series of tests were undertaken to determine the relative persistence of the compounds which fluoresce at 418 nm when excited at 403 nm in relationship to other constituents in Empire Mix crude oil during microbial degradation. For these tests 0.25 g of Empire Mix crude oil in a mineral salts medium was subjected to microbial action for varying lengths of time.

Using the fluorescence method developed and described above both samples 1 and 2 had 0.17 g of oil remaining in the system. The aliphatic profile (carried out using gas chromatography) for these two samples is shown in Figure 31. Both were essentially the same but differed from the original samples in that fewer compounds were present and those that were present were significantly reduced in quantity. The aromatic profiles (carried out using HPLC-fluorescence at 274 nm in MeOH-H_2O) for these two samples (shown in Figure 32) indicate a difference between the two samples with sample 1 having the greatest quantity of aromatics.

Using the newly developed fluorescence method, 0.24 g of oil was found in sample 3 and 0.23 g of oil was found in sample 4. The HPLC-fluorescence chromatograms carried out at 274 nm in MeOH-H_2O were likewise almost identical (Figure 33) whereas there was a definite difference in the low molecular weight aliphatics as shown in the GC tract (Figure 34).

Samples 5 and 6 both were found to have 0.19 g of oil by the newly developed method but aliphatics were virtually absent from both samples as shown in the GC trace illustrated in Figure 35. Likewise the LC-

fluorescence chromatogram taken at 274 nm using MeOH-H_2O failed to indicate oil (Figure 36).

The data from these tests indicate that the constituents in oil which fluoresce at 418 nm after excitation at 403 nm are far more persistent than the other constituents evaluated.

In still another series of test, sixteen samples of crude oil in 32 oz bottles containing mineral salts medium were incubated for six months in the presence of microorganisms. After incubation samples of the oil were analyzed for aliphatics by gas chromatography, aromatics by fluorescence using HPLC with excitation at 403 nm and emission at 418 nm. A total of twenty samples involving Empire Mix, Nigerian, Iranian, and Venezuelan crude were tested. There were no qualitative differences in the oils by any of the analytical methods employed. This was not unexpected due to the large volume of oil employed in the systems but did confirm that the compounds responsible for the results obtained by the newly developed method were not preferentially degraded by the microflora.

In still another series of tests approximately 0.25 g of Empire Mix crude oil in 50 ml of mineral salts medium was subjected to microbial action for one year. After incubation, each of the samples was shaken and 2 ml removed, extracted twice with 2 ml of hexane, and the hexane extracts evaporated to dryness under N_2, then diluted to 100 ml.

All samples showed oil by the HPLC fluorescence method developed and described in this report. Samples 70b, 71b, and 20y show some disappearance of volatile aromatic hydrocarbons (LC at 274 nm in methanol and water), while samples 72p and 19y were almost devoid of aromatics (LC at 274 nm in methanol and water). Most aliphatics in samples 70b, 71b, and 20y had disappeared (GC analysis) except for pristane and phytane. Aliphatics in samples 71p, 72p, and 19y were almost totally absent. There seems to be little doubt that the fraction of oil detected in the newly developed fluorescence method using chloroform as the solvent, excitation at 403 nm, and measuring emission at 418 nm is more persistant than most of the other oil fractions.

EVALUATION OF FACTORS EFFECTING A CONTINUOUS FLOW SYSTEM

At the time that the proposal for work to be performed under this

contract, (October, 1975), the scope of work included consideration of developing a continuous flow monitoring system. It was realized at the outset that the development of an in situ device would be doubtful in view of the problems encountered due to marine fouling. During the course of work under this contract a series of experiments were conducted to determine the applicability of making light scattering measurements of oil-water emulsions under various conditions. It was concluded, however, that light scattering is not a desirable technique for determining oil in an aqueous medium.

After development of the fluorescence method described earlier some attention was given to preprocessing the water samples prior to analysis by HPLC. The use of Bio-rad hollow fibers, fine porosity fitted glass discs and Bio-beads all failed to improve on the methods of sample preparation described in this report.

The fluorescence measurements performed using the method developed under this contract were made on oil extracts and extracts of marine organisms exposed to oil, as well as not exposed to oil. Maximum sensitivity differences between biogenic flucrescence and oil fluorescence were observed at an excitation of 403 nm and an emission of 418 nm. This suggests that if one wishes to monitor petroleum derived fluorescence in the absence of biogenically derived fluorescence, a 403-418 fluorescence system would be optimal.

At the time this project was underway, a report appeared by Gary S. Keys and B. F. Hochheimer (Sea Technology 18, 24 (1977)) entitled "The Design of a Simple Fluorometer for Underwater Detection of RThodamine Dye." Although the instrument was designed for use with a red dye, there is no reason that the instrument could not be modified to detect petroleum derived fluorescence by changing the filters. It would be ideal if the excitation energy could be isolated as near as possible to 403 nm and the emission energy as near as possible to 418 nm.

The instrument designed by Keys and Hochheimer takes into consideration the practical problems associated with reducing a theoretical fluorescence system into actual practice. This fluorometer could be used as a tow for short periods of time (hrs), rather than continuously, in order that biofouling not become a problem.

SUMMARY

A chemical method of detecting petrogenic hydrocarbons in water and sediment has been developed. The method involves the use of high pressure liquid chromatograph using chloroform as the solvent and measuring fluorescence in the range of 418 nm after excitation at 403 nm. The method is capable of detecting petroleum hydrocarbons in the presence of overwhelming amounts of biological material. It has been demonstrated that the fractions of oil which fluoresce at 418 nm after excitation at 403 nm are among the most persistant fractions in crude oil.

Of the six crude oils employed in this investigation, Saudi Arabian was the least responsive to the above technique and yet is still detectable in quantities of less than 0.1 μg and easily quantifiable at 1 μg. Under these circumstances oil is detectable in the ppb range using small to moderate sized water samples (one liter or less).

It has been reported (Brown, 1977) that an oil concentration of 5.5 ppb in water would be harmless to the biological community and, therefore, the newly developed method is capable of detecting oil at concentrations biological harm.

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APPENDIX A

Figures

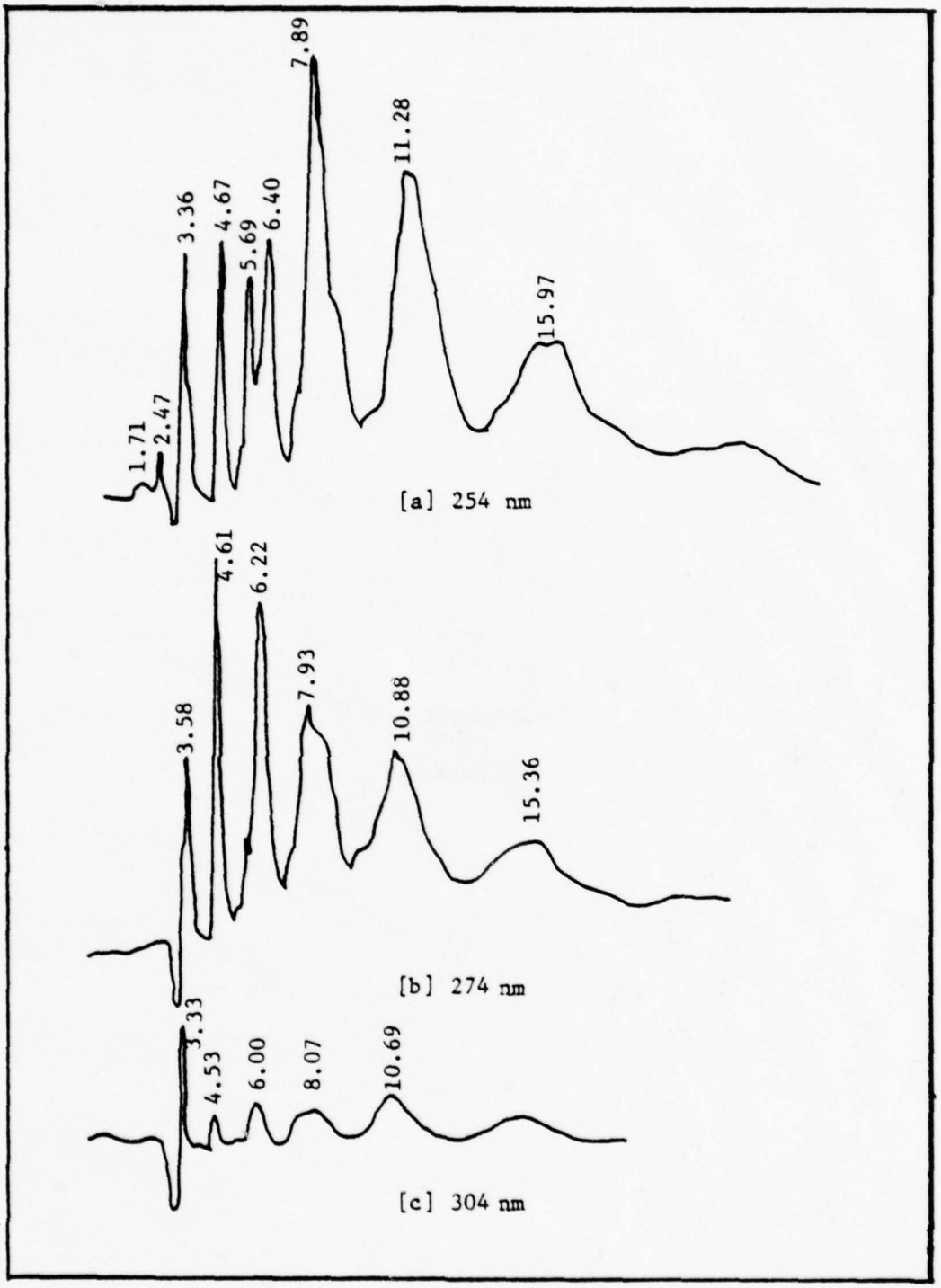


Figure 1. HPLC Chromatogram of 100 µg Samples of Empire Mix Crude Using a UV Detector at Varying Wavelengths. (Numbers above peaks are retention times in minutes.)

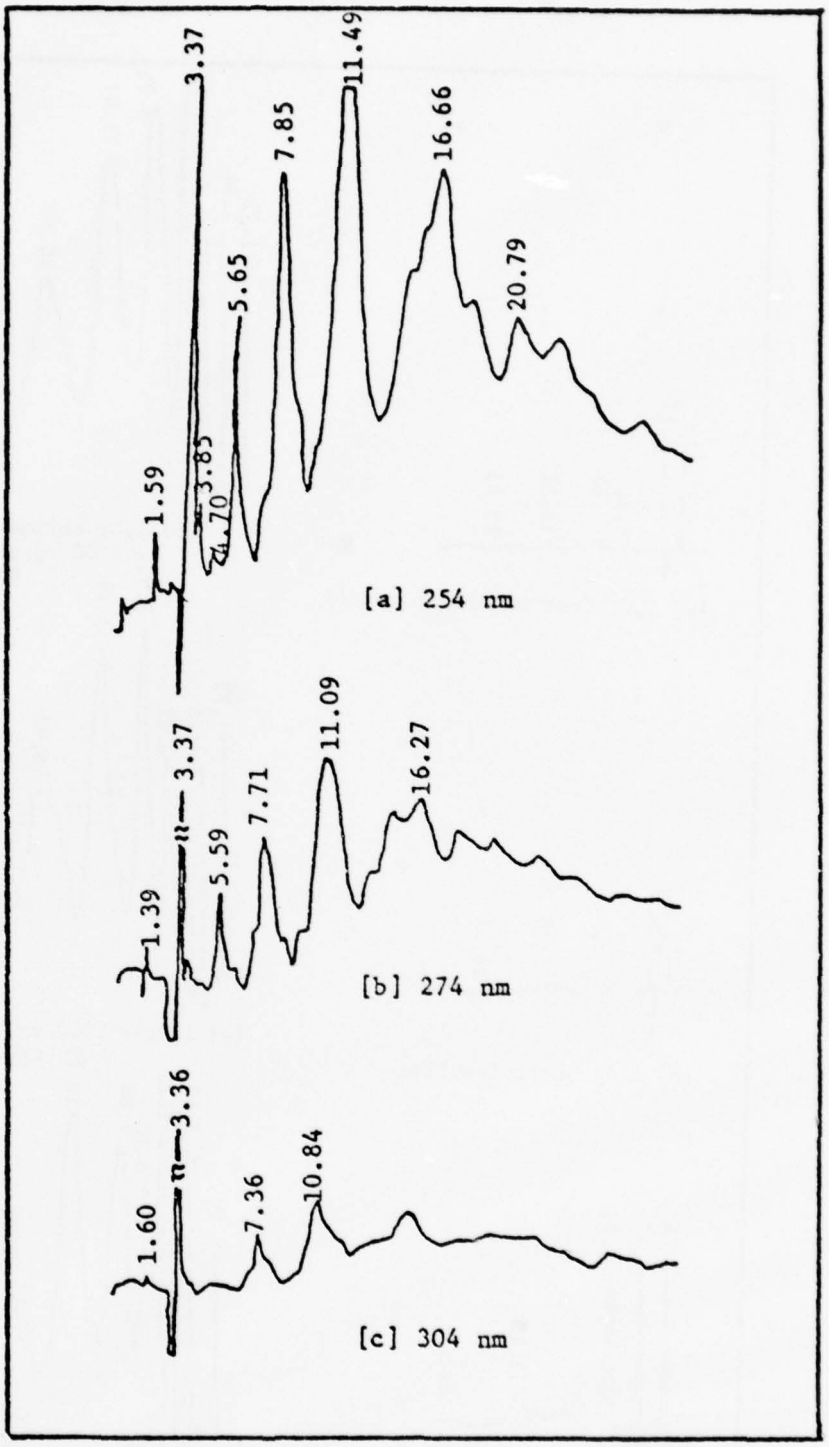


Figure 2. HPLC Chromatograms of Control Oysters (10 g) Using a UV Detector at Varying Wavelengths. (Numbers above peaks are retention times in minutes.)

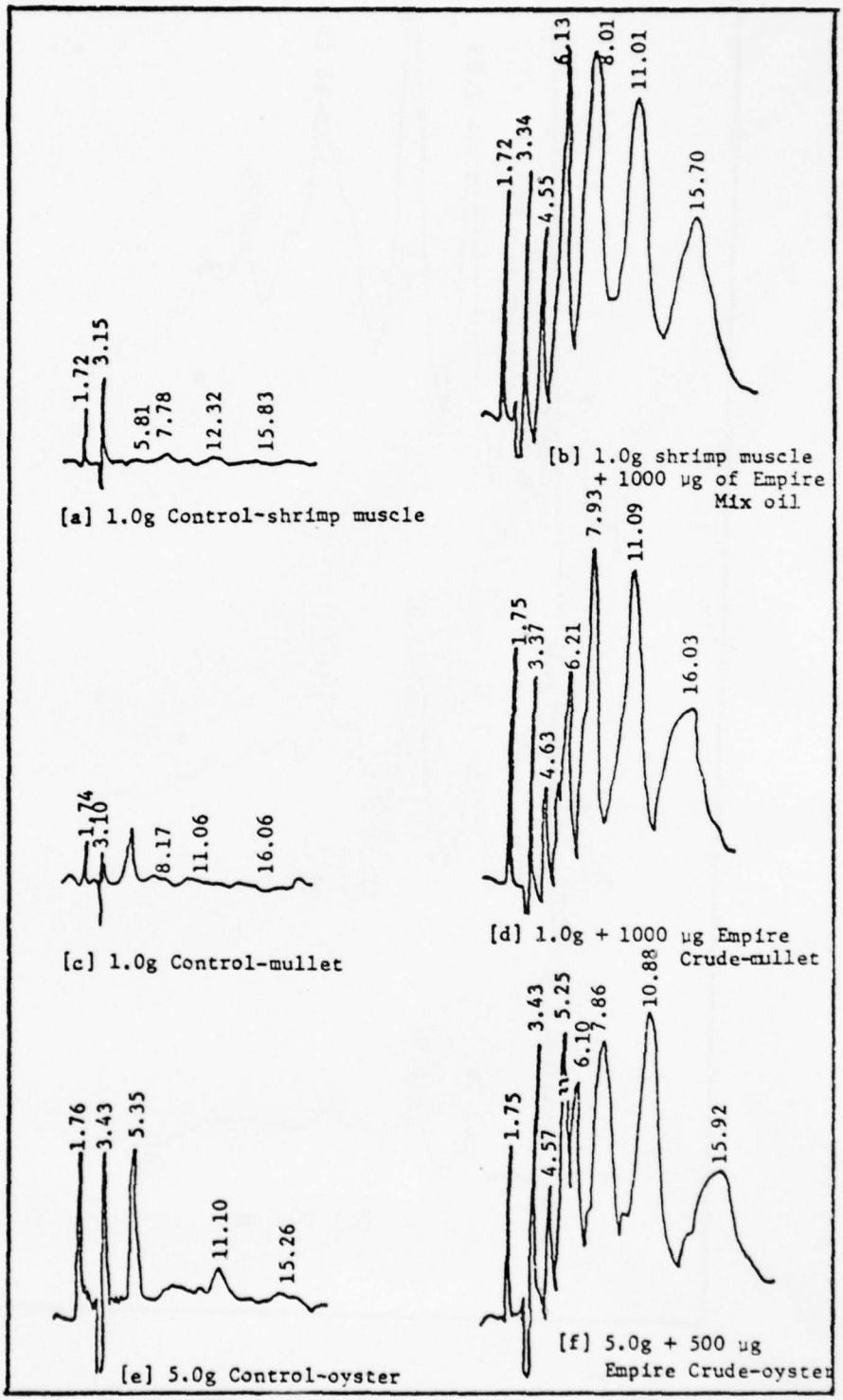


Figure 3: HPLC Chromatograms of Control and Spiked Organisms Using a UV Detector at a Wavelength of 277 nm. (Numbers above peaks are retention times in minutes.)

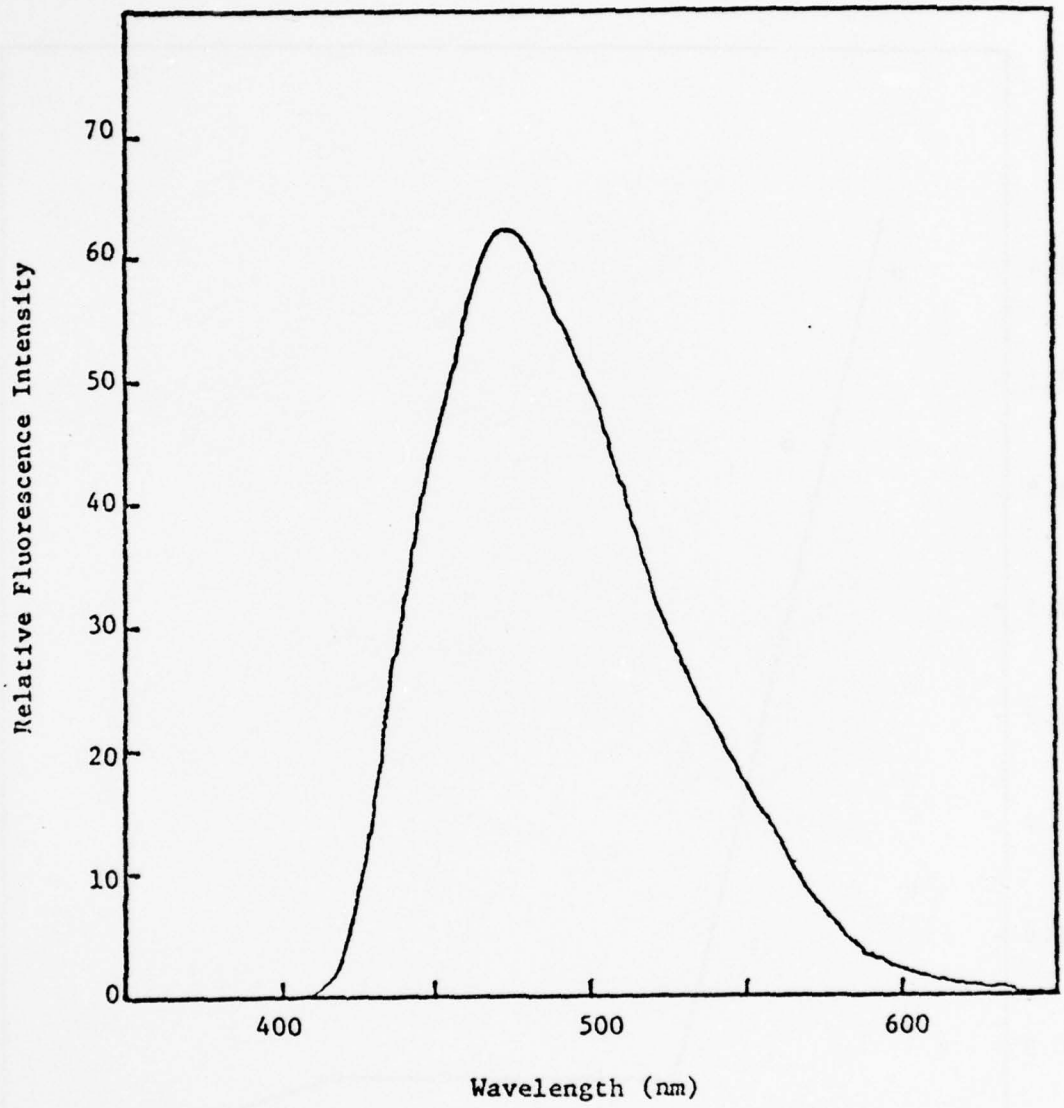


Figure 4. Emission Spectrum of Chloroform Fraction of Saudi Arabian Crude Excitation at 432 nm. (Sample was diluted approximately 1000-fold with hexane prior to scan.)

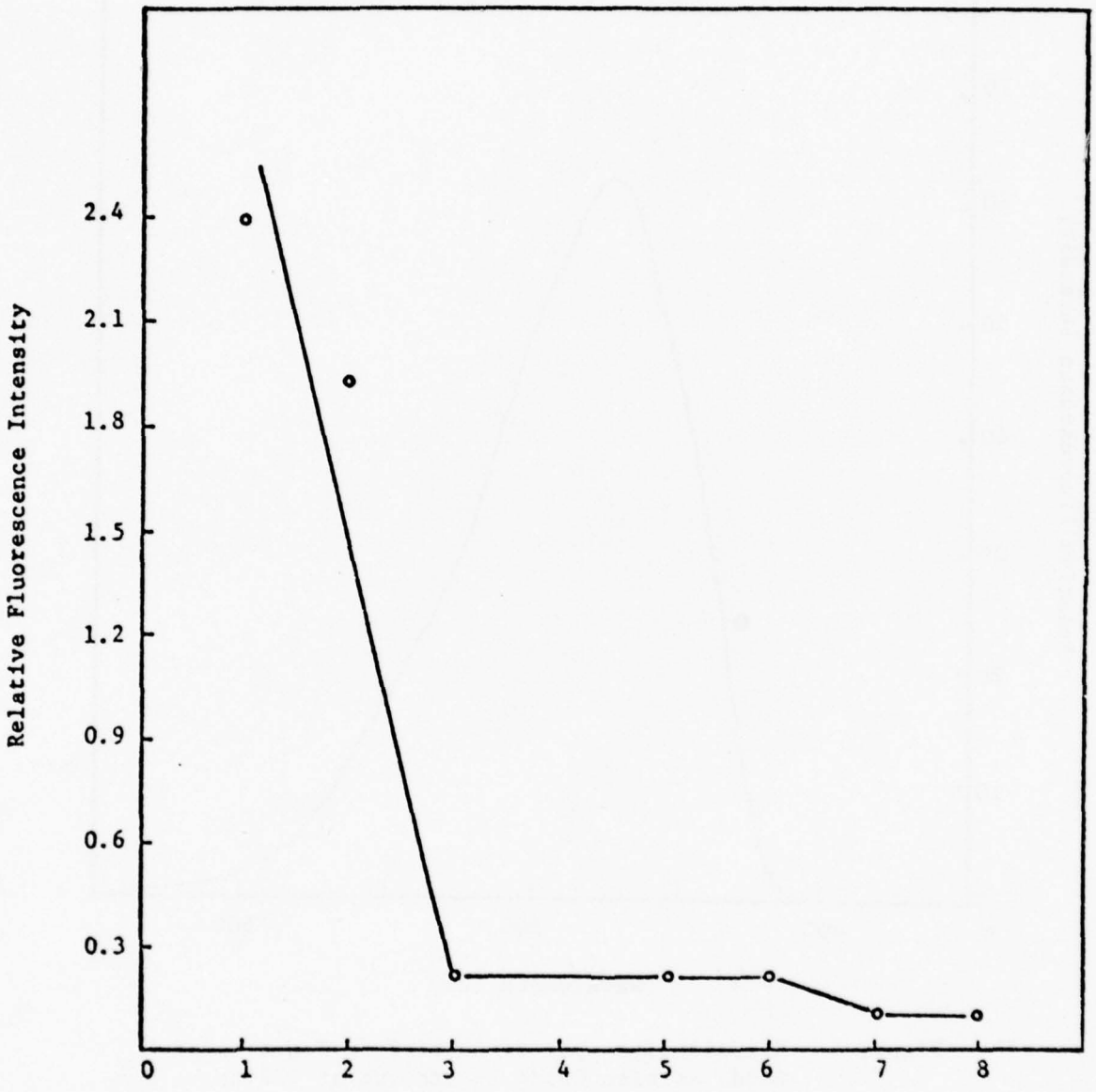


Figure 5: Decrease in Fluorescence as a Function of Dilution for Hexane Fraction of Empire Crude. (Instrumental settings used were 300 nm excitation and 418 nm emission on hexane dilutions of original sample.)

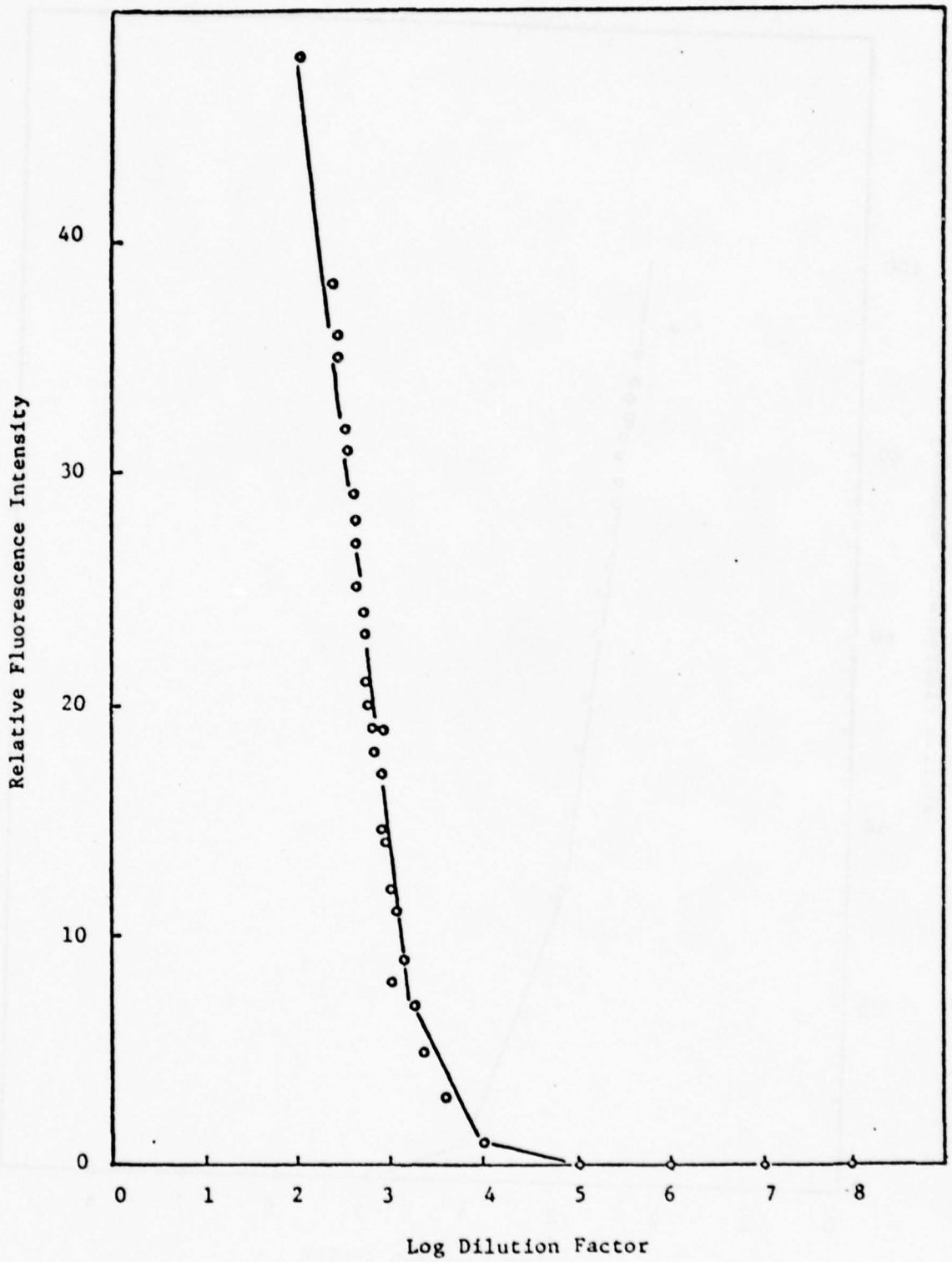


Figure 6: Decrease in Fluorescence as a Function of Dilution for Heavy Crude Fraction of Iranian Crude. (Instrumental settings used were 303 nm excitation and 357 nm emission in hexane dilutions of original sample.)

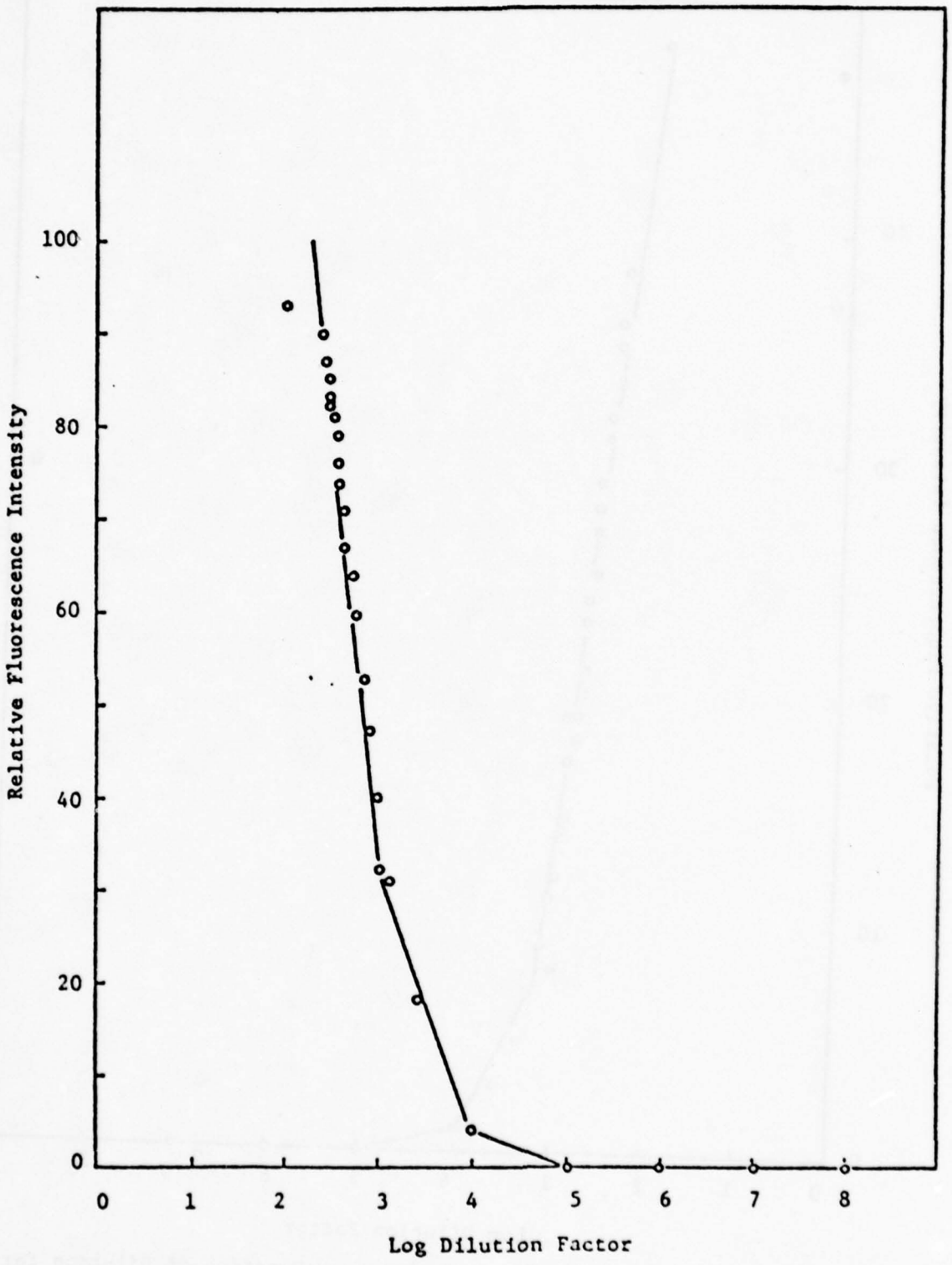


Figure 7: Decrease in Fluorescence as a Function of Dilution for Benzene Fraction of Nigerian Crude. (Instrumental settings used were 328 nm excitation 375 nm emission for hexane dilutions of original sample.)

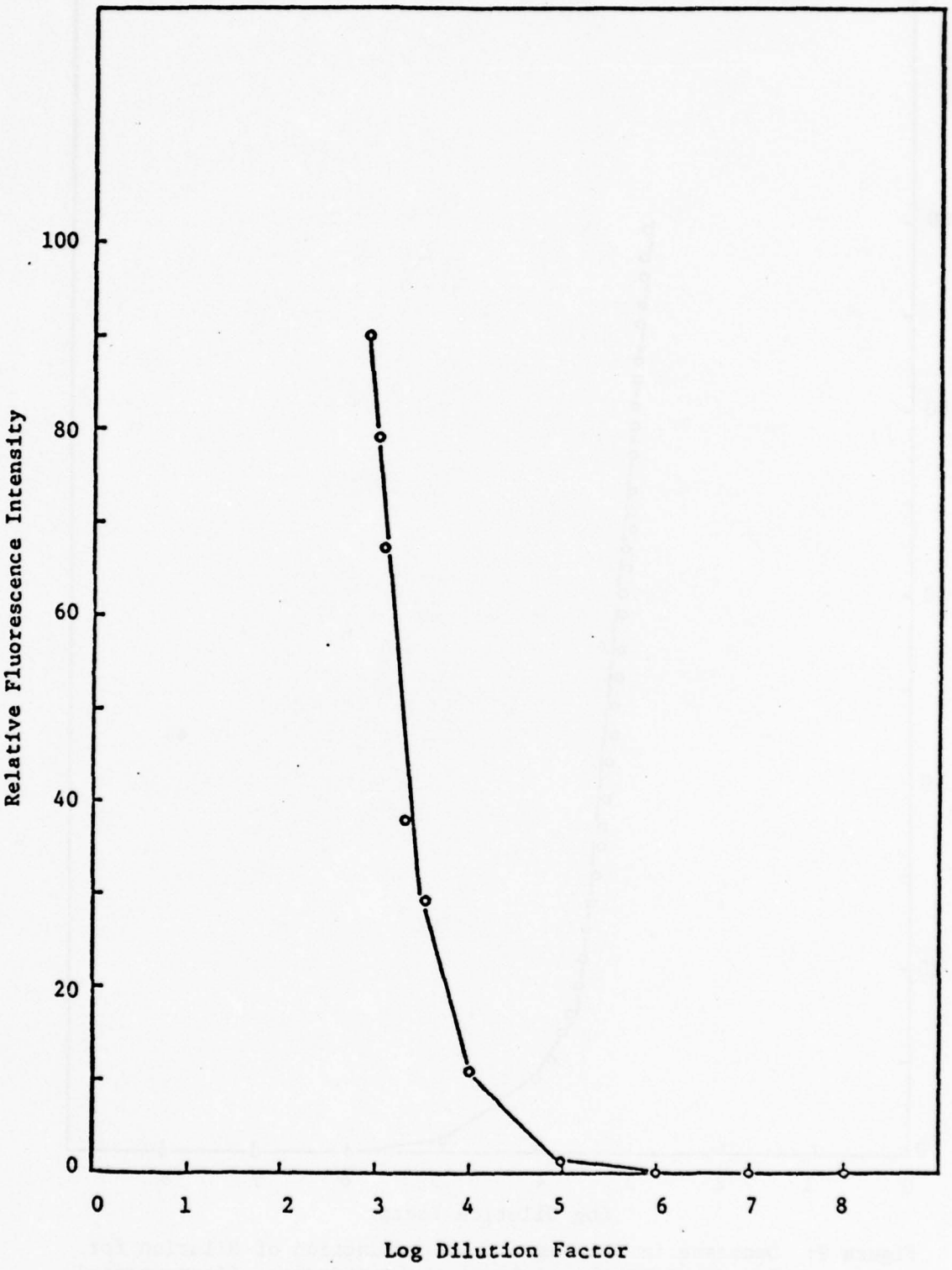


Figure 8: Decrease in Fluorescence as a Function of Dilution for Methanol Fraction of Saudi Arabian Crude. (Instrumental settings used were 425 nm excitation and 464 nm emission for hexane dilution of original sample.)

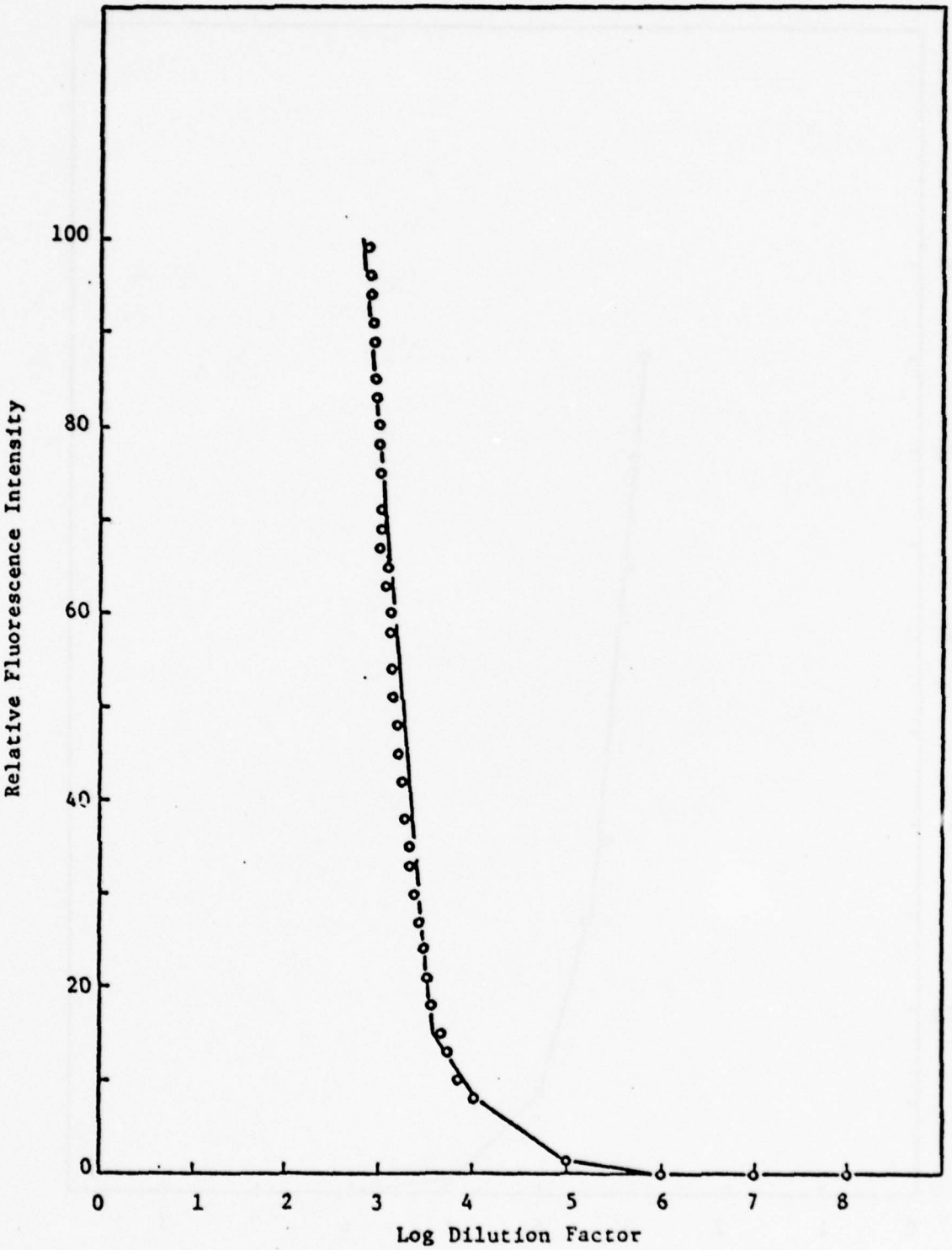


Figure 9: Decrease in Fluorescence as a Function of Dilution for Benzene Fraction of Saudi Arabian Crude. (Instrumental settings used were 403 nm excitation and 435 nm emission for hexane dilutions or original sample.)

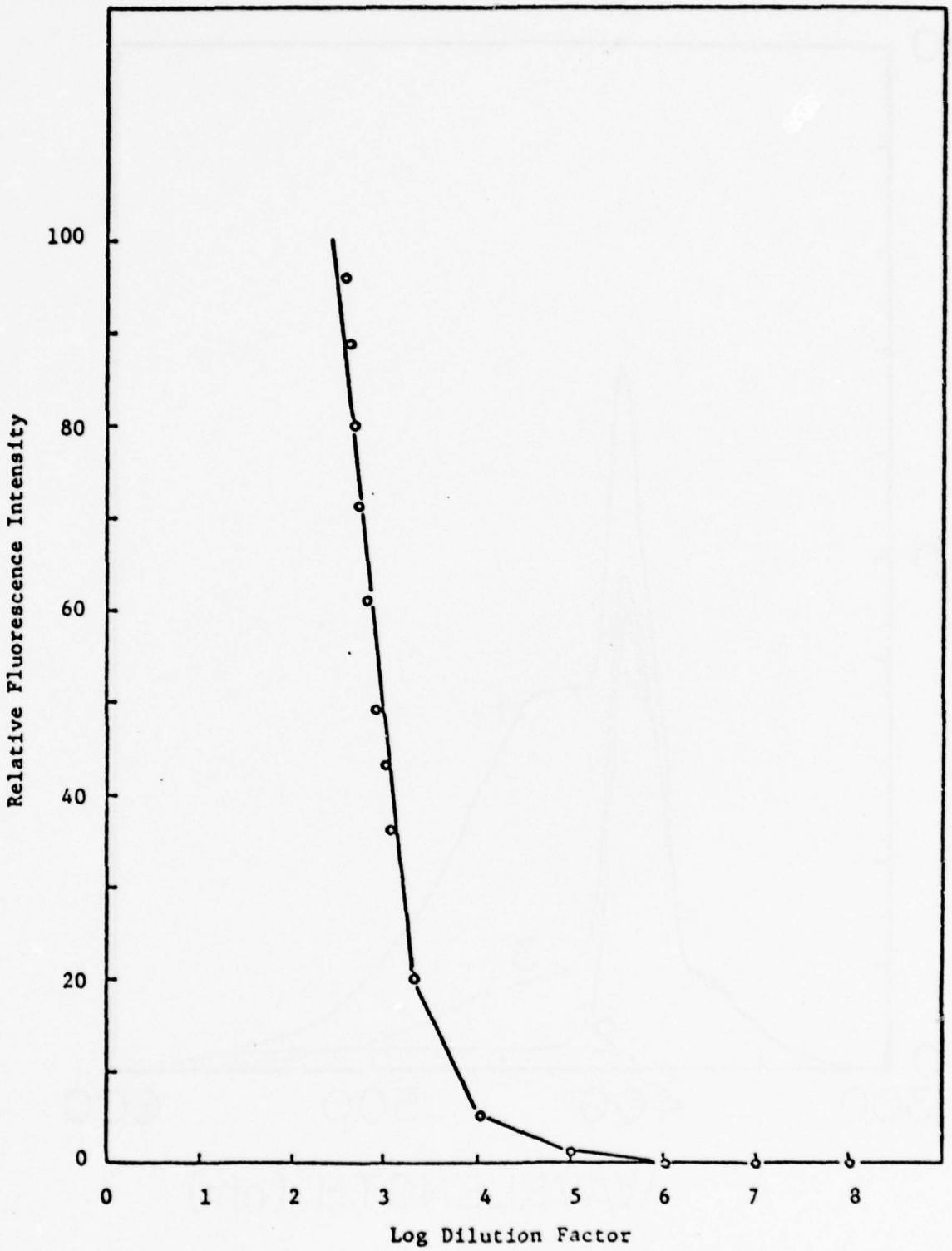


Figure 10: Decrease in Fluorescence as a Function of Dilution for Chloroform Fraction of Saudi Arabian Crude. (Instrumental settings used were 432 nm excitation and 472 nm emission for hexane dilutions of original sample.)

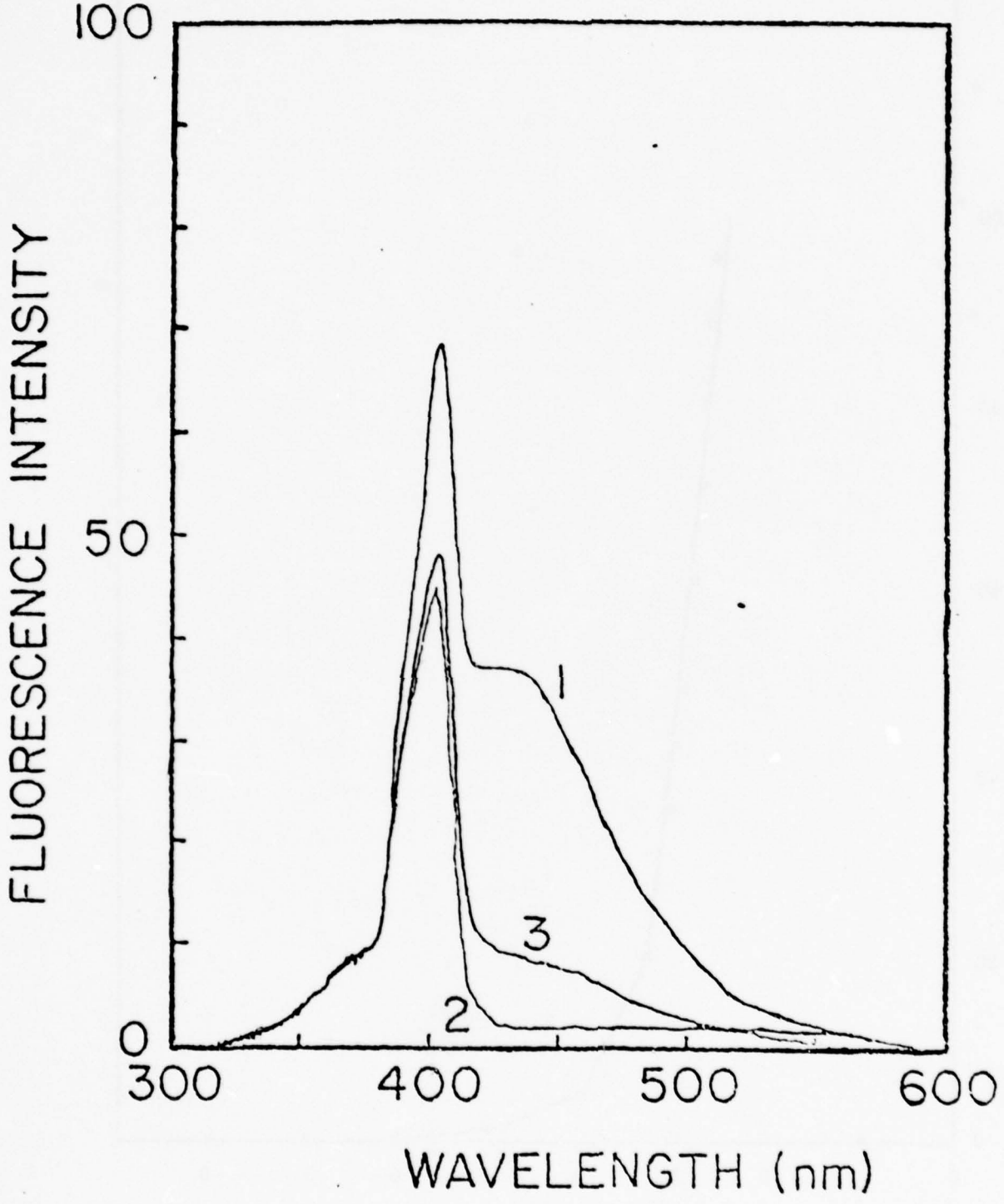


Figure 11: Fluorescence Emission Spectrum of Saudi Arabian Crude Oil (Curve 1), Shrimp Tissue Only (Curve 2), and Shrimp Tissue Spiked with Saudi Arabian Crude Oil (Curve 3).

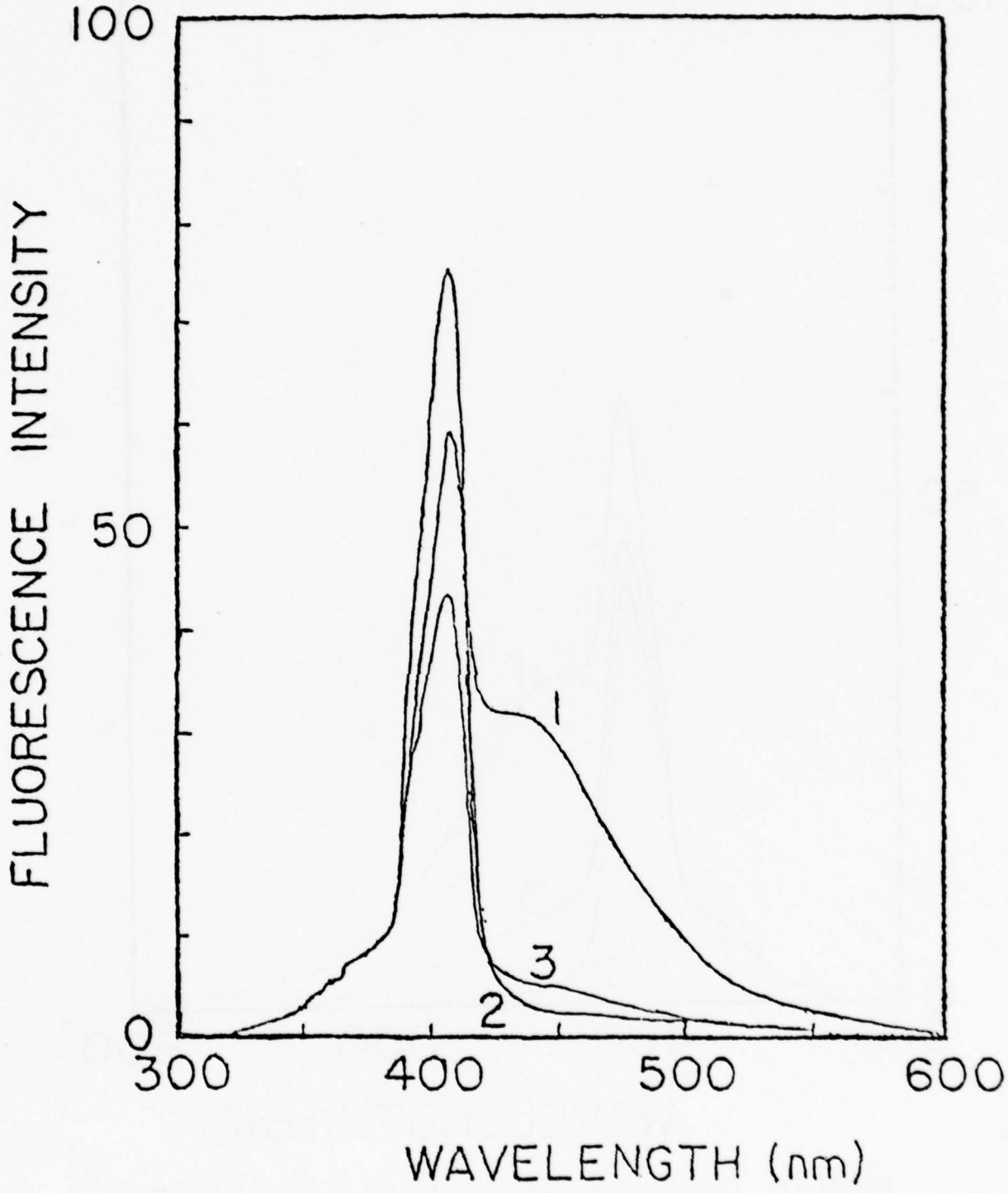


Figure 12: Fluorescence Emission Spectrum of Saudi Arabian Crude Oil (Curve 1), Oyster Tissue Only (Curve 2), and Oyster Tissue Spiked with Saudi Arabian Crude Oil (Curve 3).

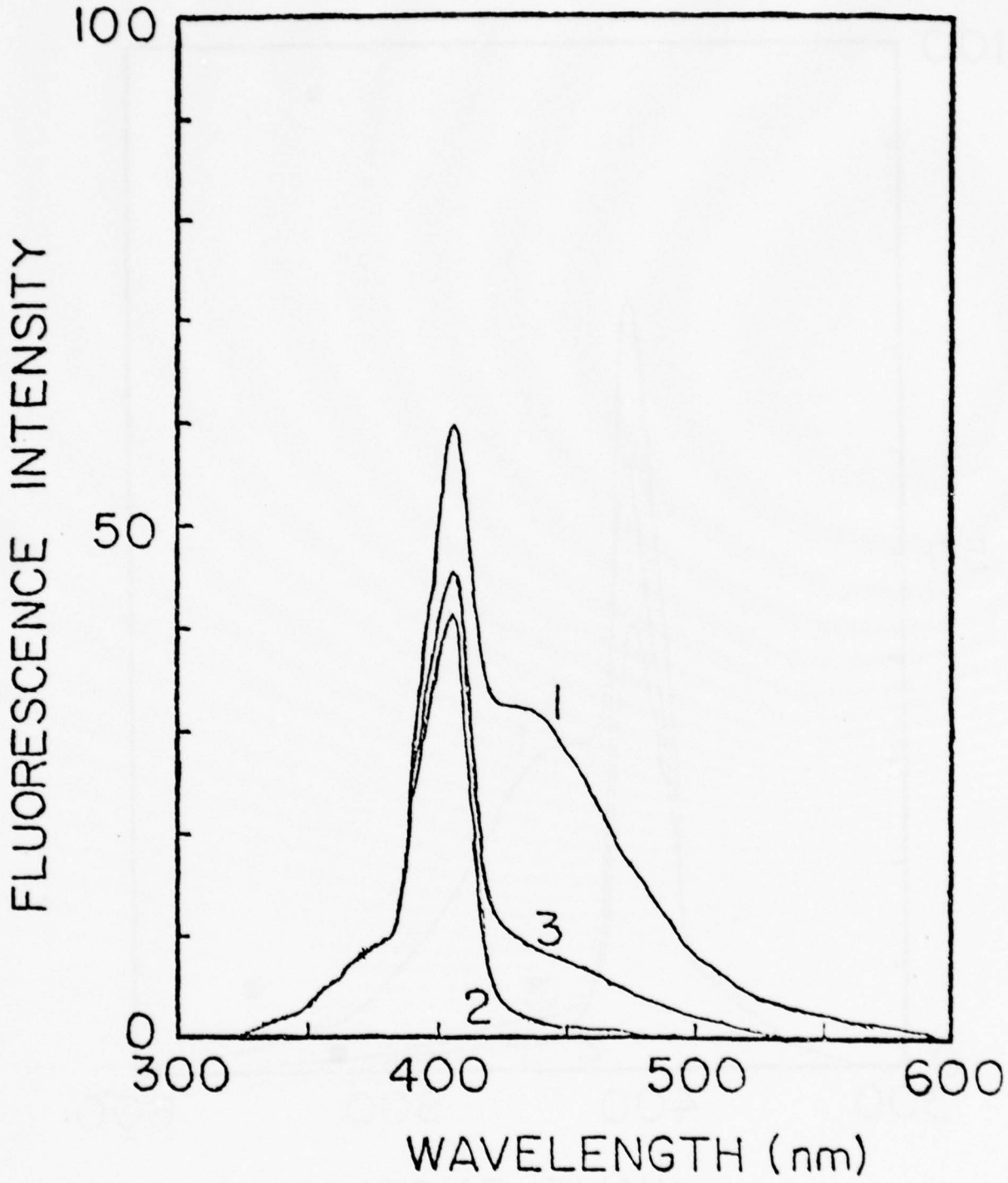


Figure 13: Fluorescence Emission Spectrum of Saudi Arabian Crude Oil (Curve 1), Mullet Tissue Only (Curve 2), and Mullet Tissue Spiked with Saudi Arabian Crude Oil (Curve 3).

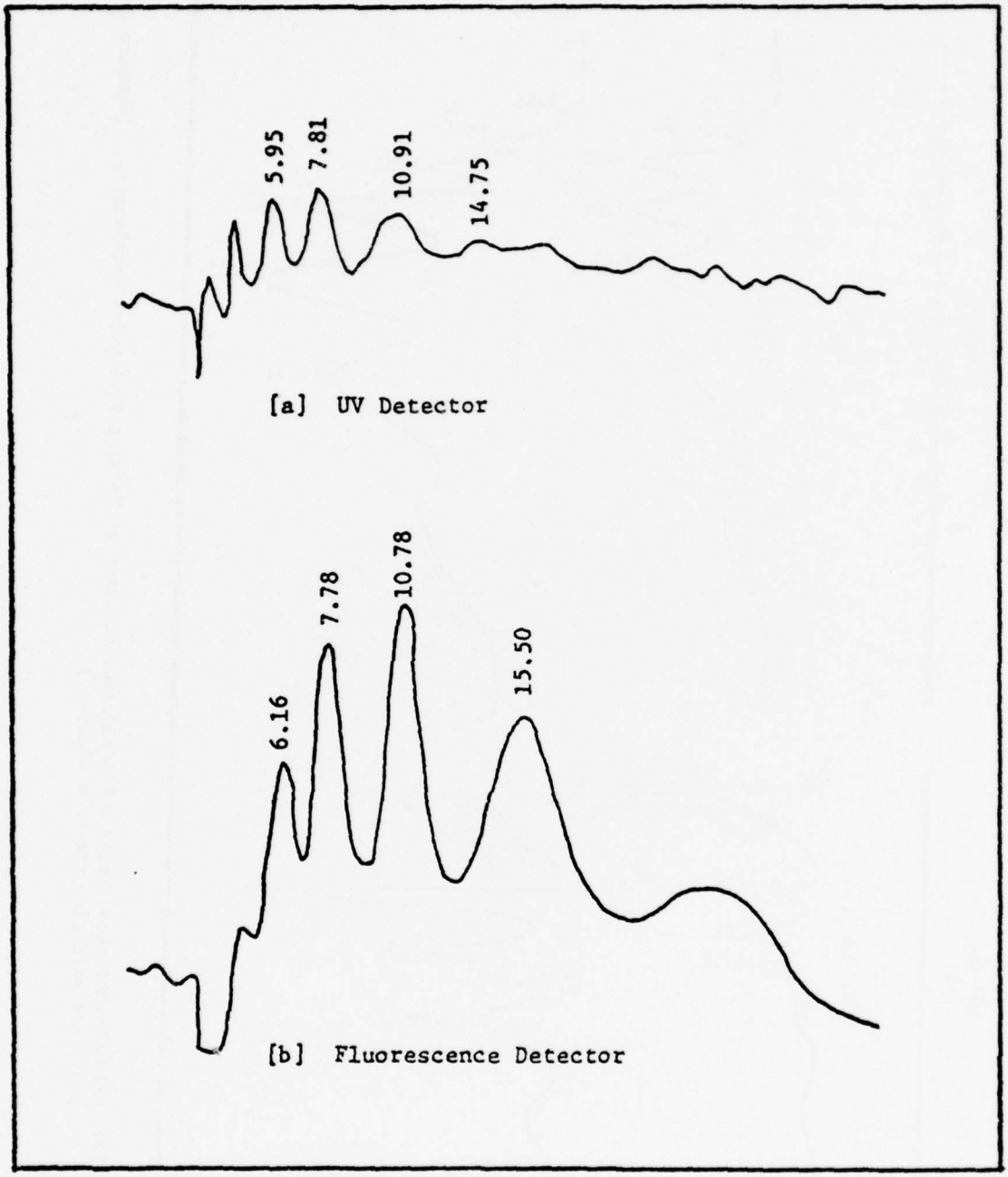


Figure 14: HPLC Chromatograms of the Benzene-Soluble Fraction of 100 µg of Empire Mix Crude Oil with the UV and Fluorescence Detectors. (Numbers above peaks are retention times in minutes.)

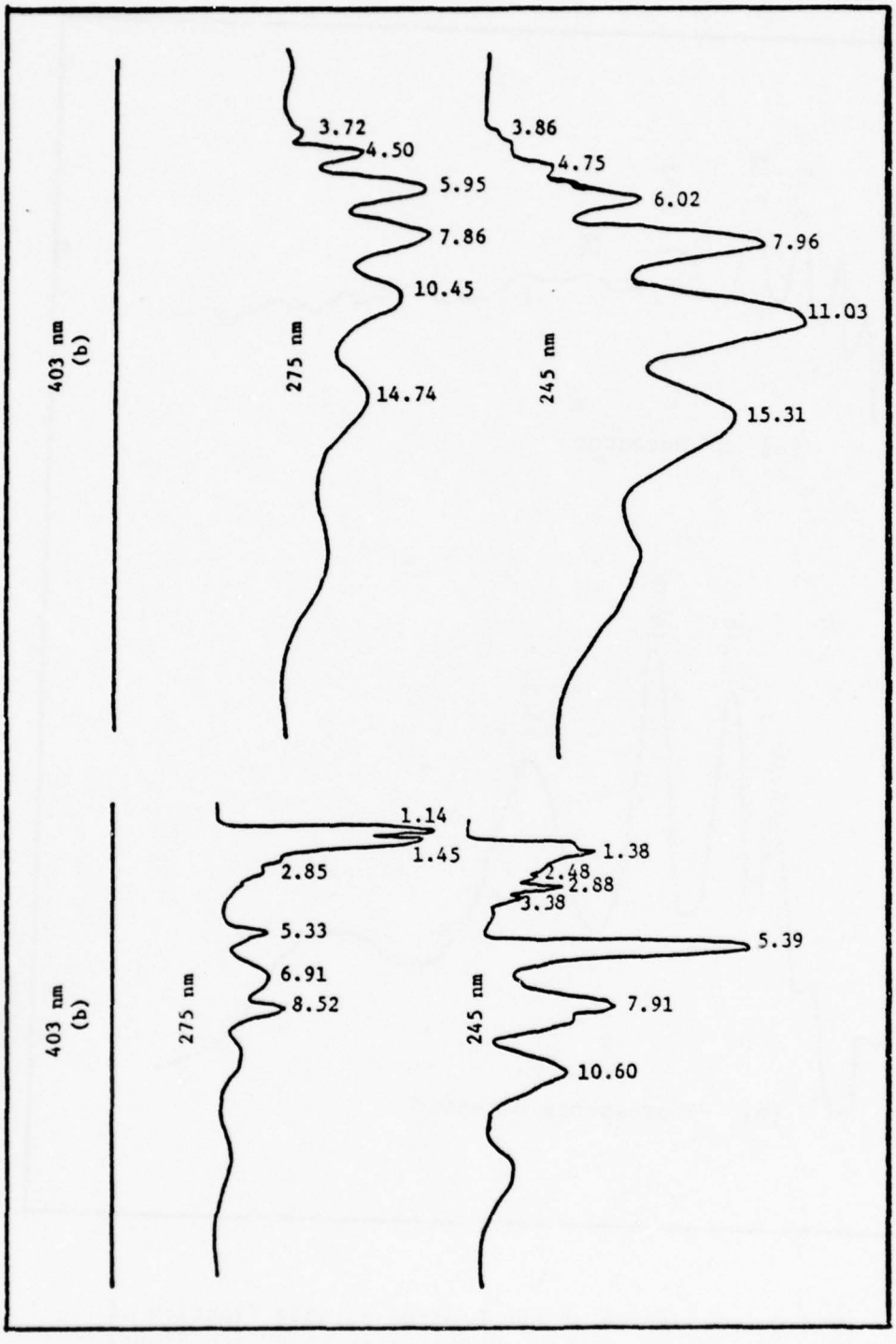


Figure 15: HPLC Chromatograms Using a Fluorescence Detector at Varying Excitation Wavelengths. (Numbers above peaks are retention times in minutes.)

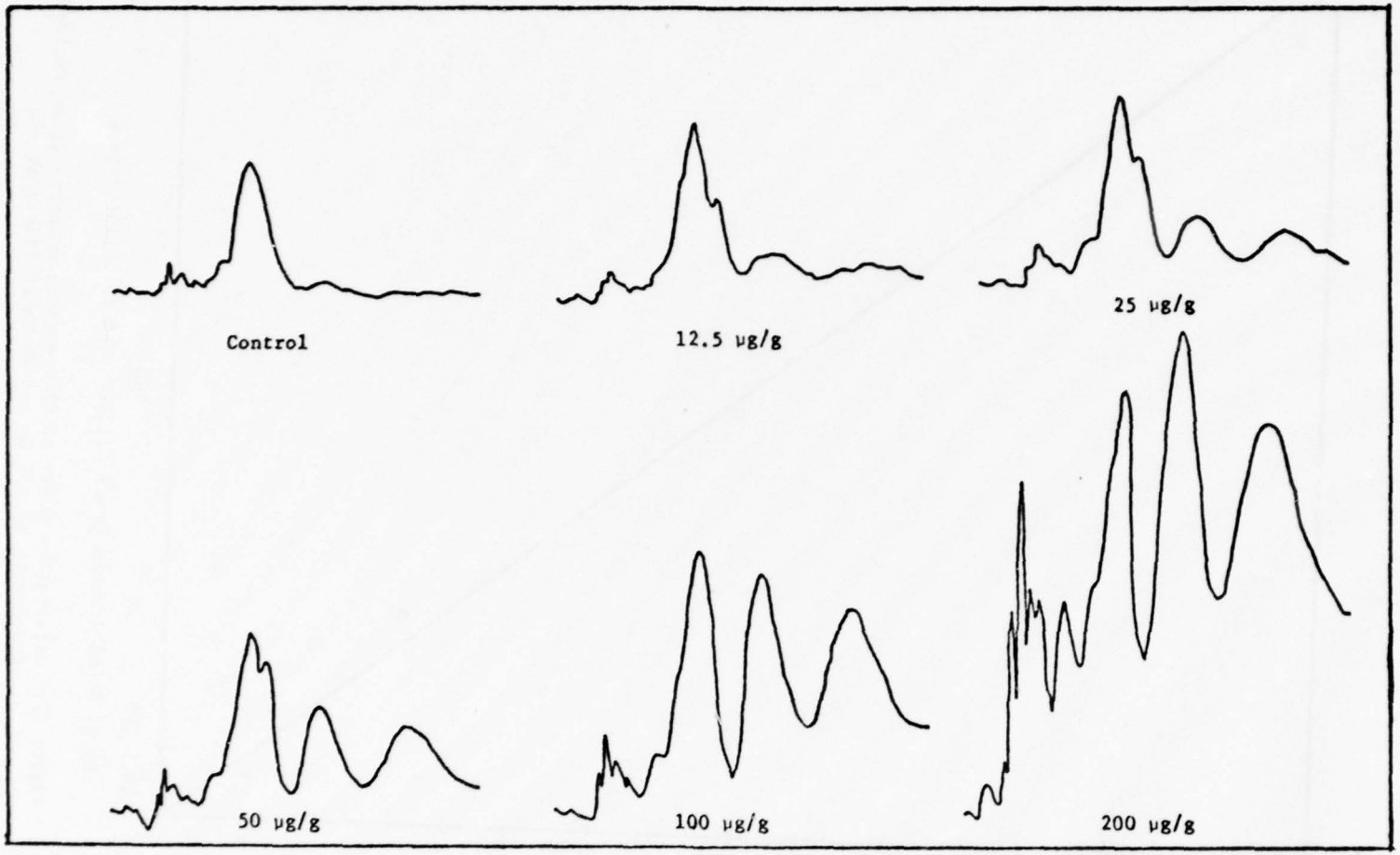


Figure 16: HPLC Chromatograms of Control Oysters and Oysters Spiked with Varying Amounts of Saudi Arabian Crude Using a Fluorescence Detector at a Excitation Wavelength of 274 nm and Emission Filter KV 370. (Numbers above peaks are retention times in minutes.)

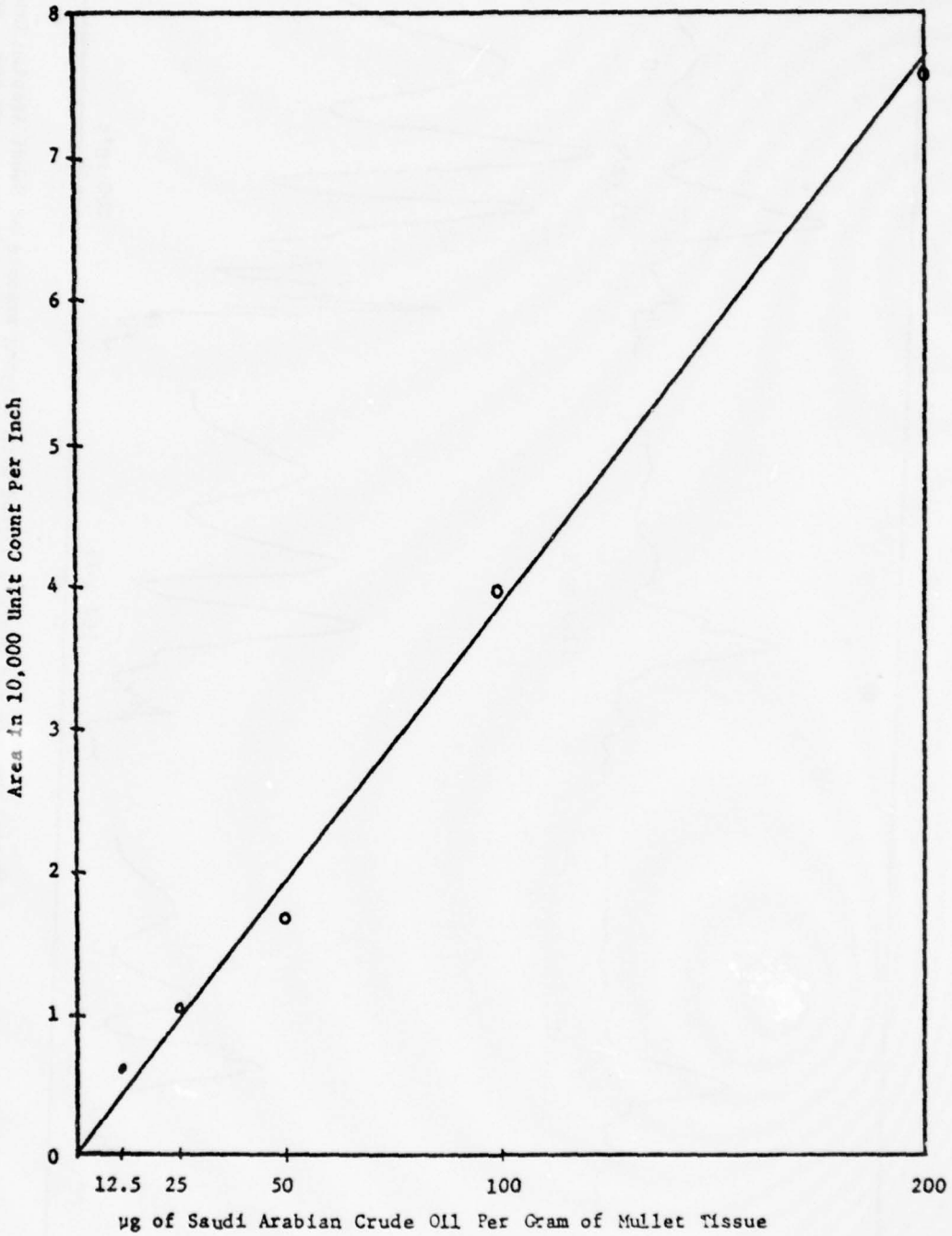
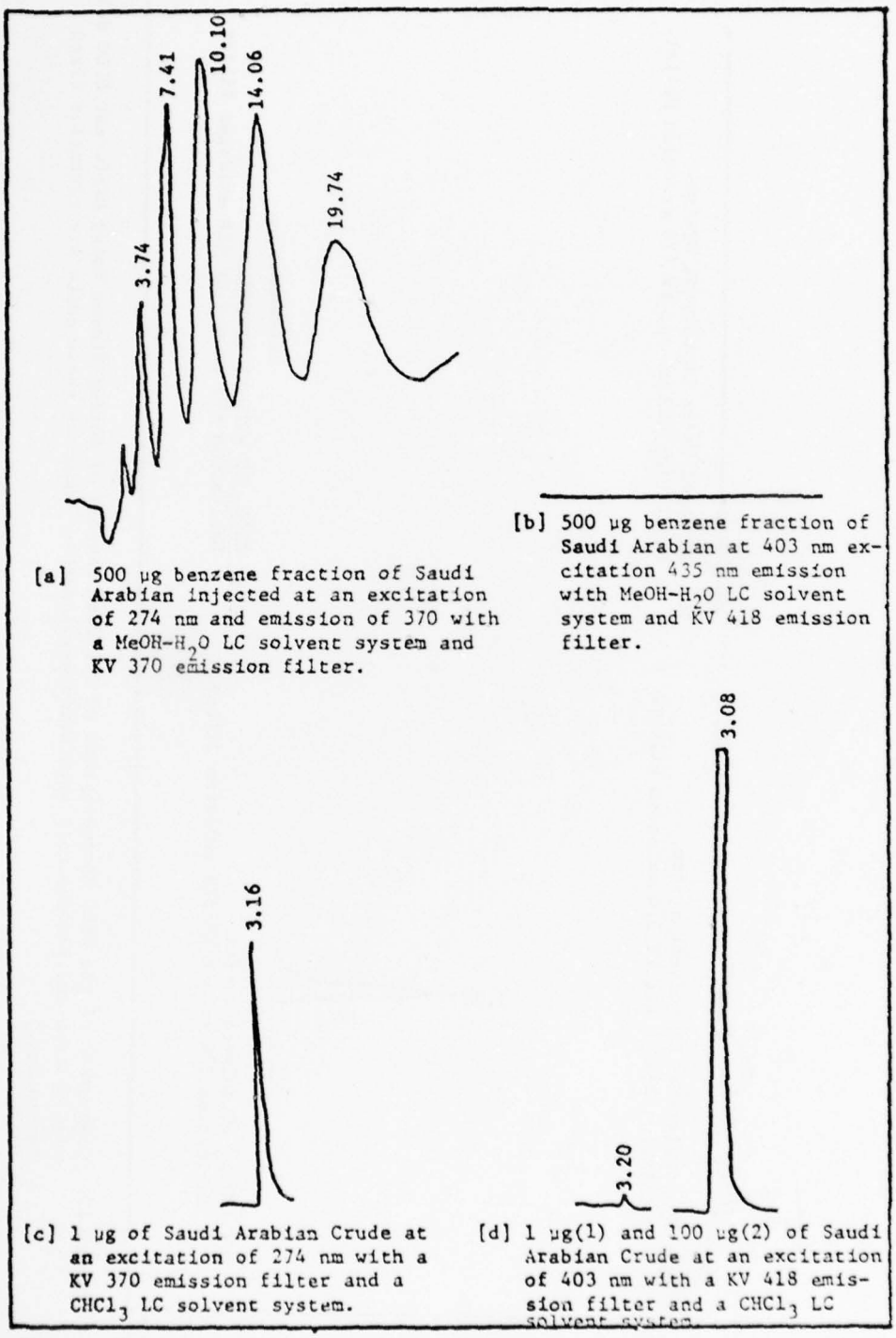


Figure 17: Calibration Curve of Fluorescence Detector at an Excitation Wavelength of 274 nm and Emission Filter KV 370.



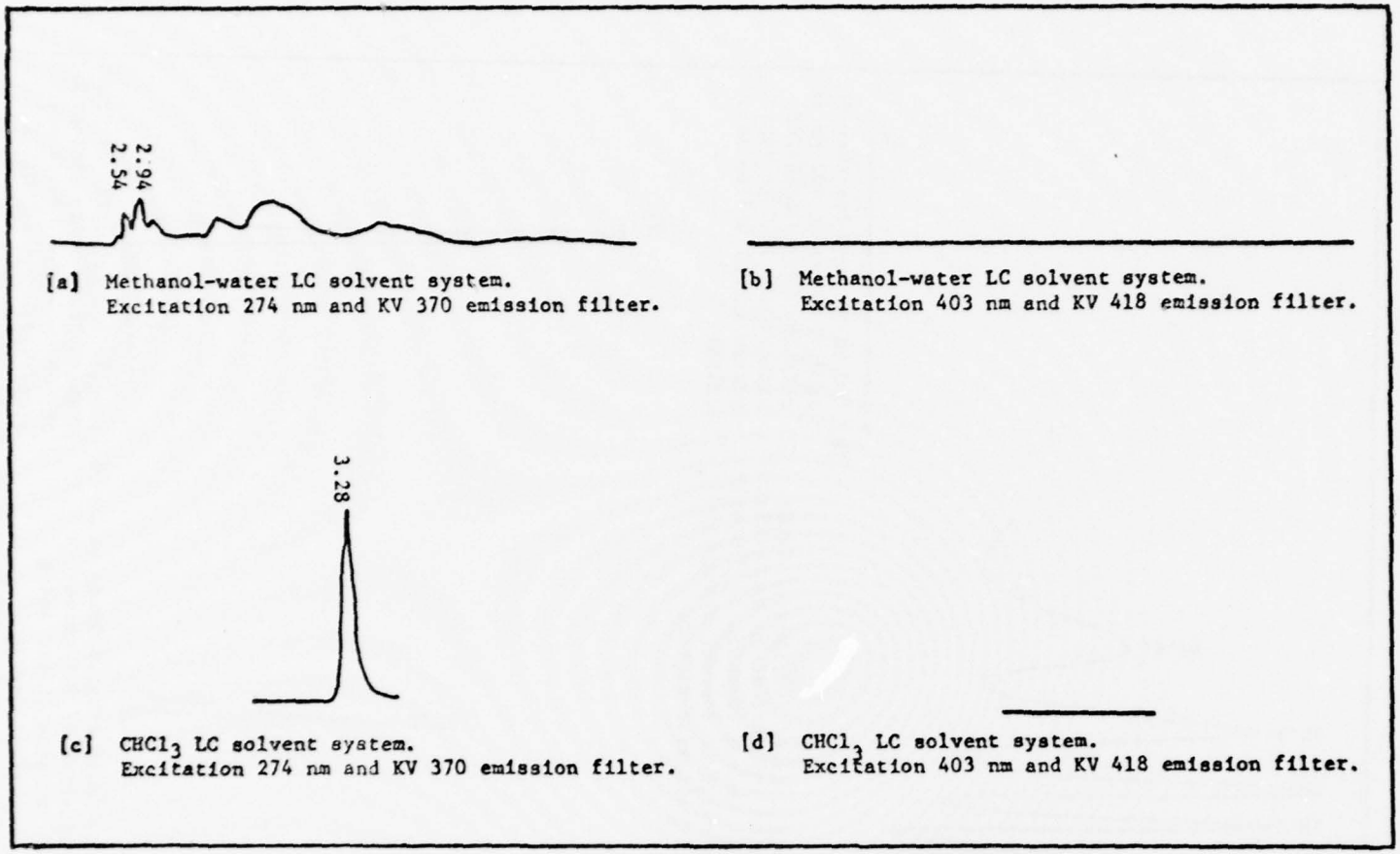
[a] 500 µg benzene fraction of Saudi Arabian injected at an excitation of 274 nm and emission of 370 with a MeOH-H_2O LC solvent system and KV 370 emission filter.

[b] 500 µg benzene fraction of Saudi Arabian at 403 nm excitation 435 nm emission with MeOH-H_2O LC solvent system and KV 418 emission filter.

[c] 1 µg of Saudi Arabian Crude at an excitation of 274 nm with a KV 370 emission filter and a $CHCl_3$ LC solvent system.

[d] 1 µg(1) and 100 µg(2) of Saudi Arabian Crude at an excitation of 403 nm with a KV 418 emission filter and a $CHCl_3$ LC solvent system.

Figure 18: Comparison of the HPLC Chromatograms Using Different HPLC Solvent Systems and Fluorescence Excitation Wavelengths (Numbers above peaks are retention times in minutes).



[a] Methanol-water LC solvent system.
Excitation 274 nm and KV 370 emission filter.

[b] Methanol-water LC solvent system.
Excitation 403 nm and KV 418 emission filter.

[c] $CHCl_3$ LC solvent system.
Excitation 274 nm and KV 370 emission filter.

[d] $CHCl_3$ LC solvent system.
Excitation 403 nm and KV 418 emission filter.

Figure 19: Comparison of the HPLC Chromatograms of One Gram Samples of Shrimp Tissue Using Different HPLC Solvent Systems and Fluorescence Excitation Wavelengths. (Numbers above peaks are retention times in minutes.)

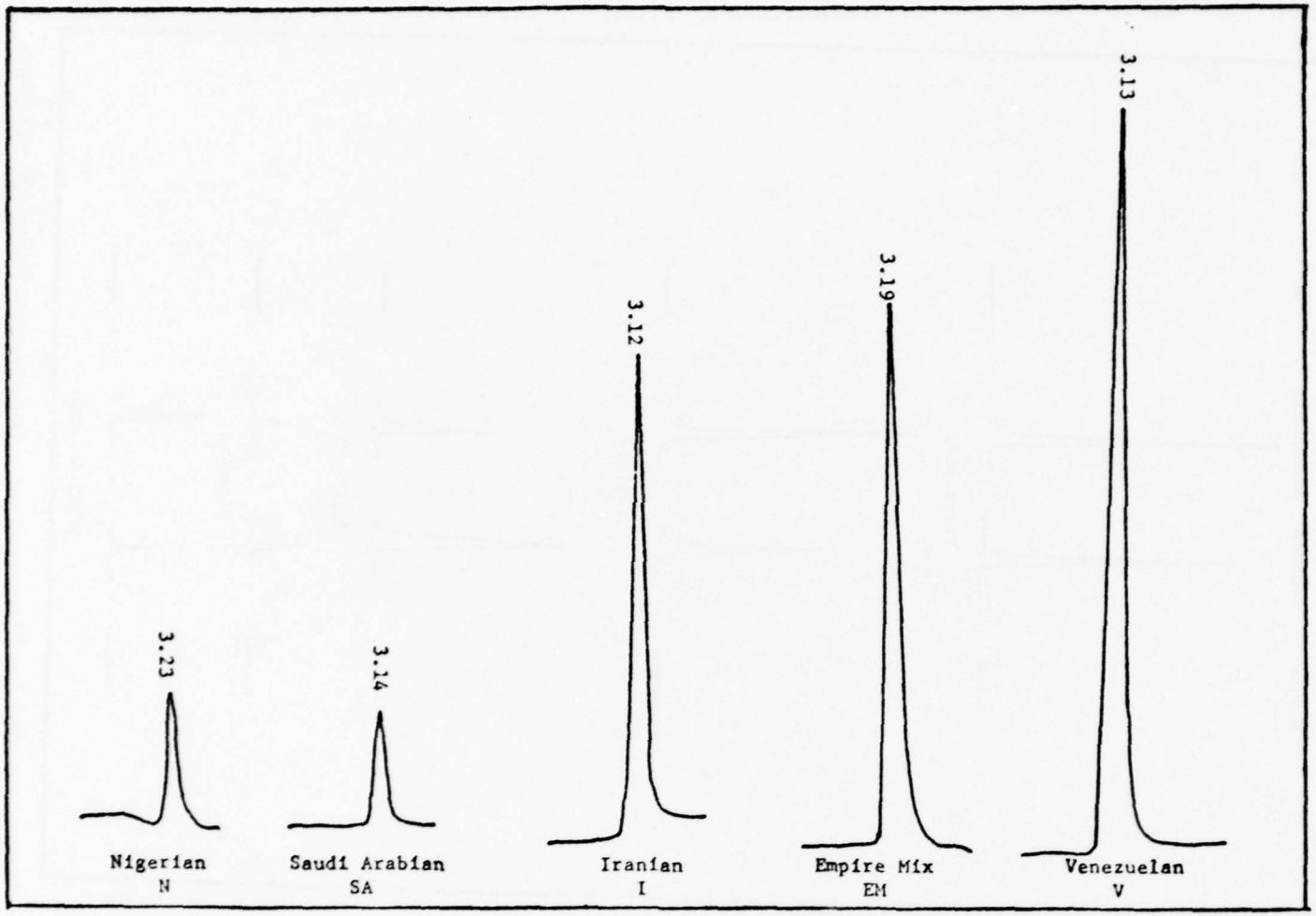


Figure 20: HPLC Chromatograms of the Benzene Eluate of Crude Oils with a Fluorescence Detection System at an Excitation Wavelength of 403 nm and with a KV 418 Emission Filter. (Numbers above peaks are retention times in minutes.)

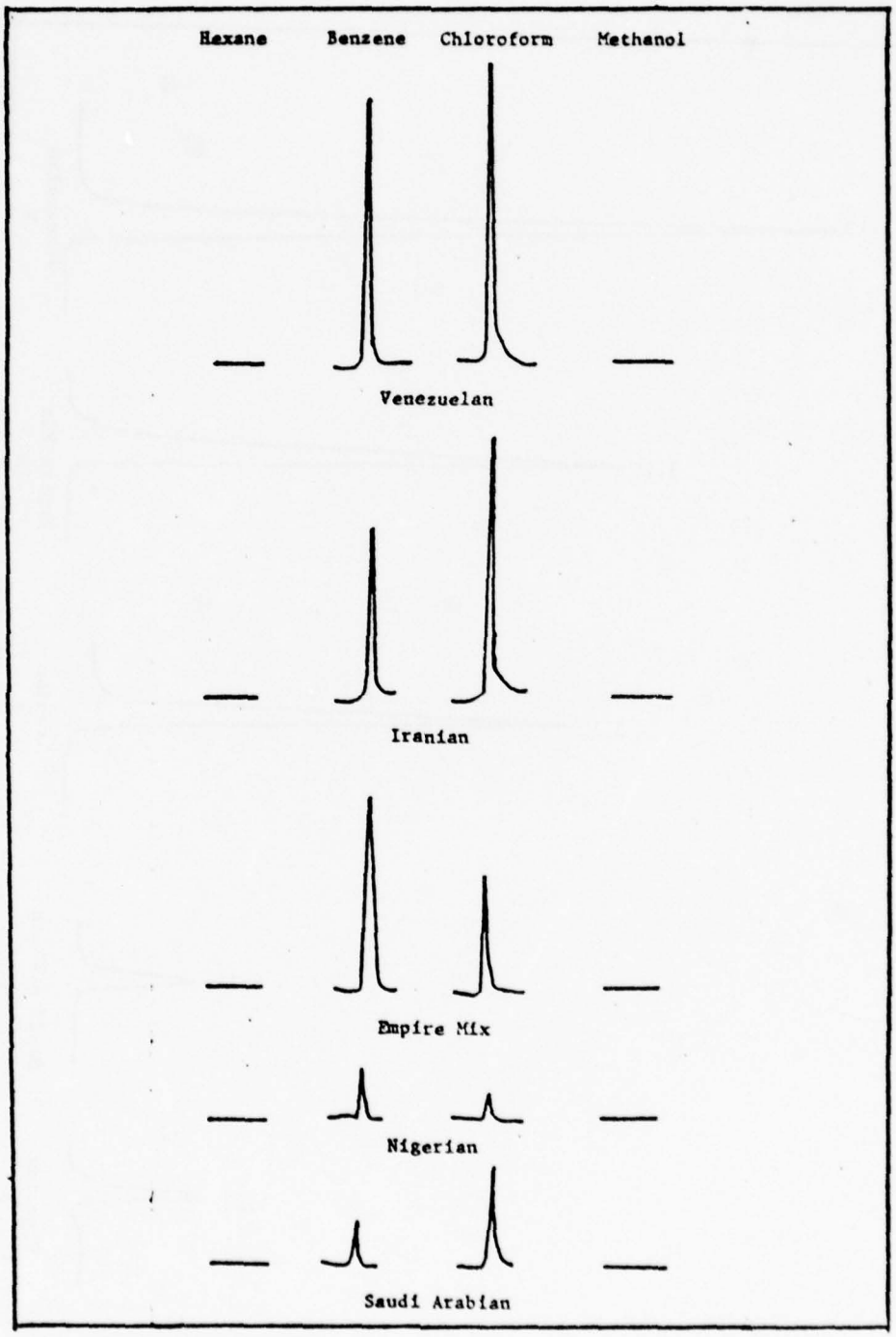


Figure 21: HPLC Chromatograms of the Hexane, Benzene, Chloroform, and Methanol Eluates of Crude Oils with a Fluorescence Detector at an Excitation Wavelength of 403 nm and with a KV 418 Emission Filter (Peak heights are relative to concentration of fluorescing material eluting at 3.18 $\pm$ 0.50 minutes.)

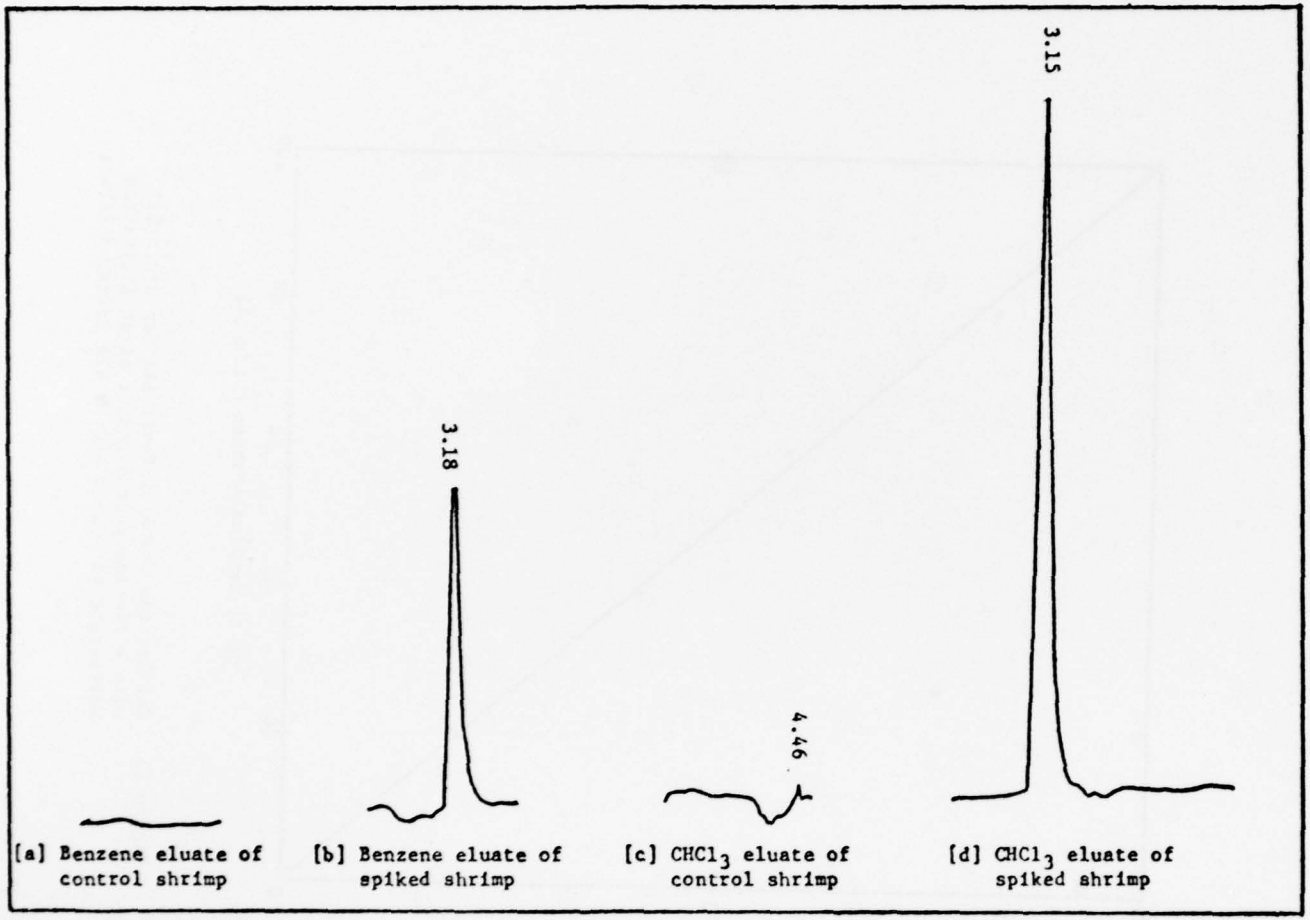


Figure 22: HPLC Chromatograms of the Benzene and Chloroform Eluates of Control Shrimp and Shrimp Spiked with Saudi Arabian Crude using a Fluorescence Detector at an Excitation Wavelength of 403 nm and with KV 418 Emission Filter (Numbers above peaks are retention times in minutes.)

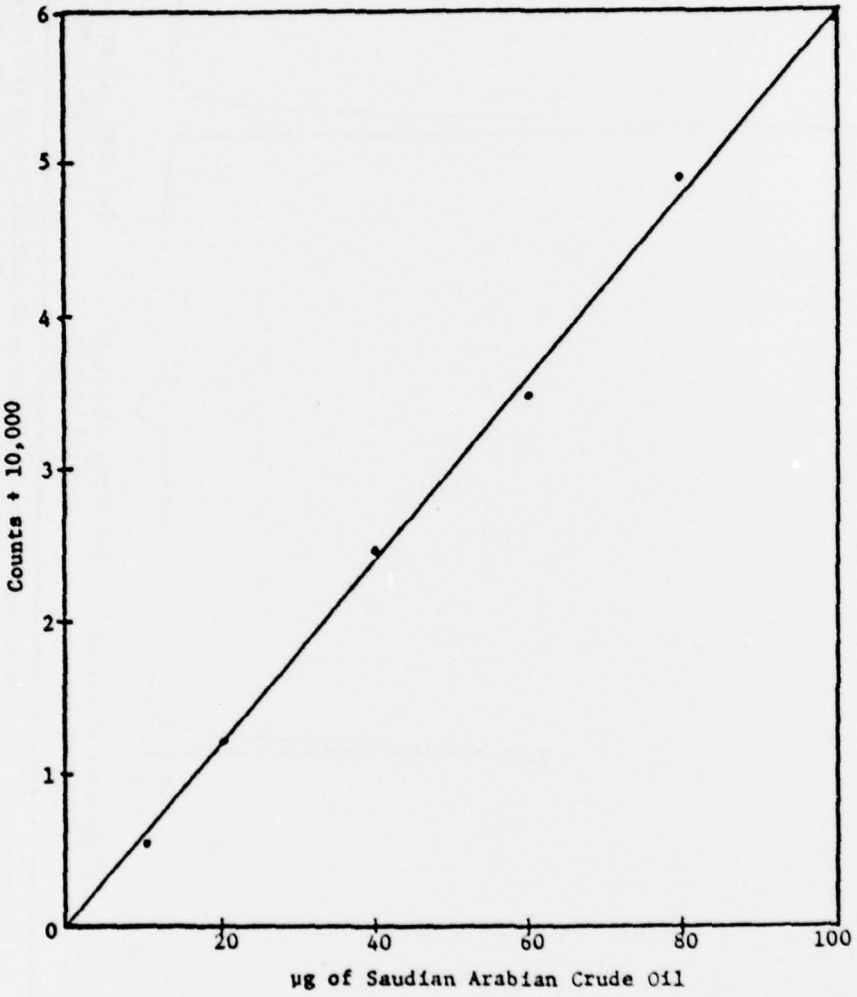


Figure 23. Calibration Graph of Saudi Arabian Crude Oil with a Fluorescence Detector at an Excitation Wavelength of 403 nm and KV 418 Emission Filter.

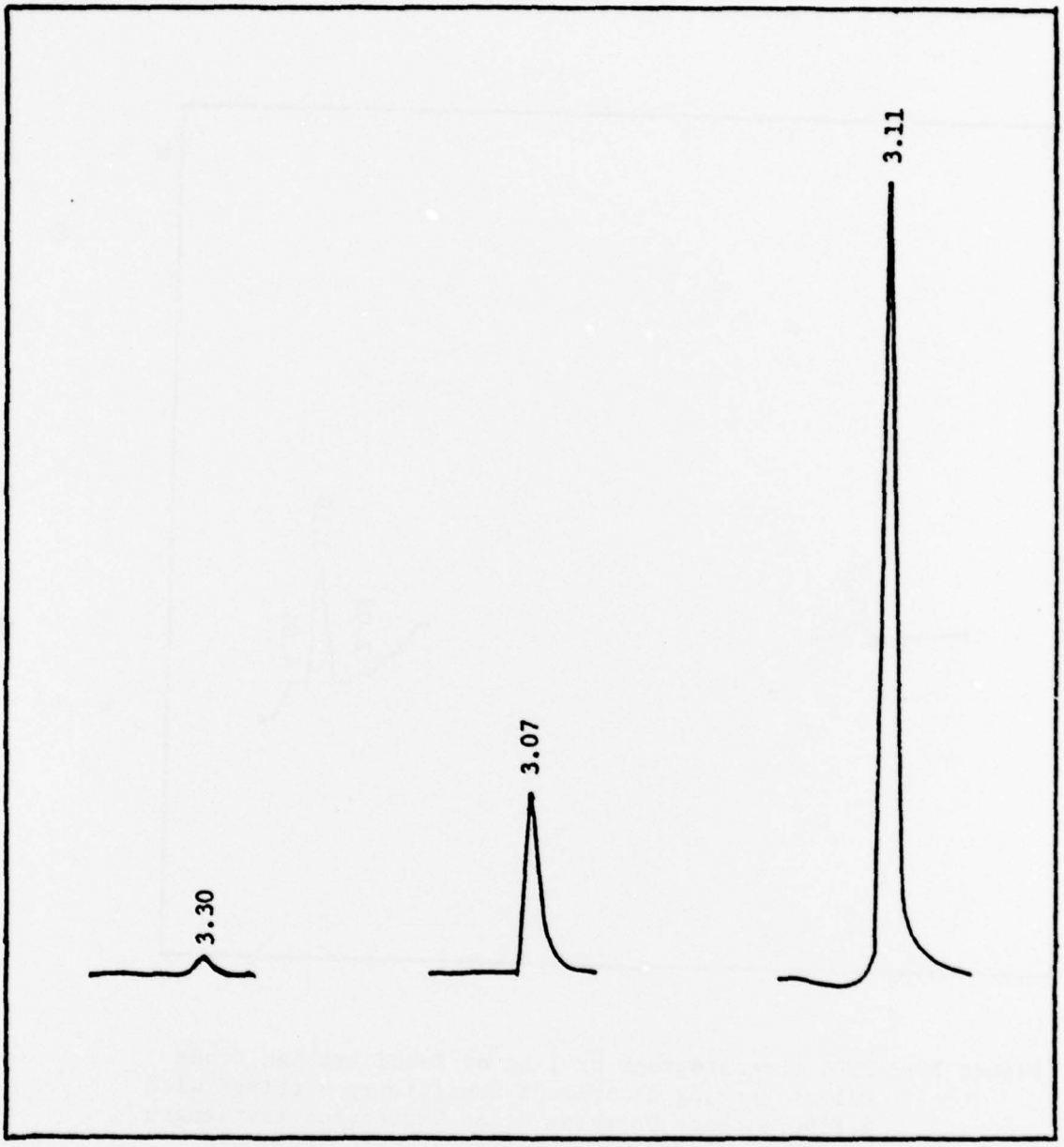


Figure 24: HPLC Chromatograms of 1 µg, 10 µg, and 100 µg of Saudi Arabian Crude with a Fluorescence Detector at an Excitation Wavelength of 403 nm and KV 418 Emission Filter. (Numbers above peaks are retention times in minutes.)

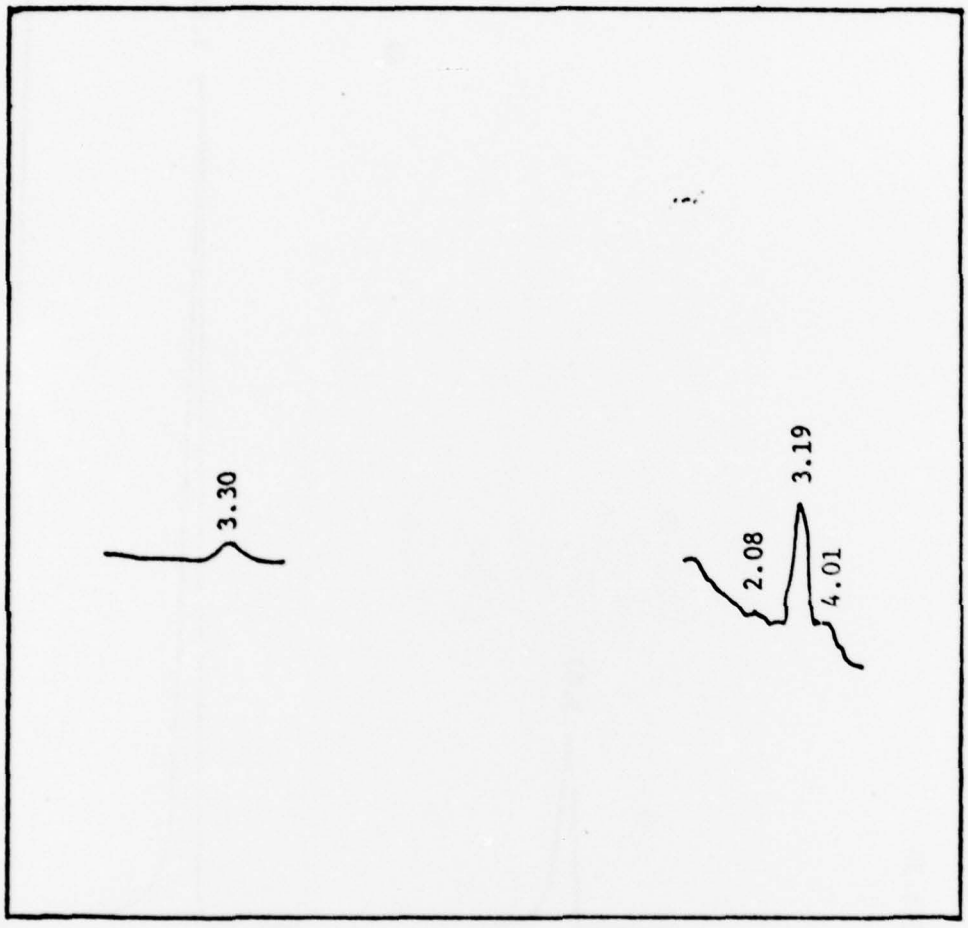


Figure 25: HPLC Chromatograms of 1 µg of Saudi Arabian Crude Oil at Varying Instrument Sensitivity Settings with a Fluorescence Detector at an Excitation Wavelength of 403 nm and KV 418 Emission Filter. (Numbers above peaks are retention times in minutes.)

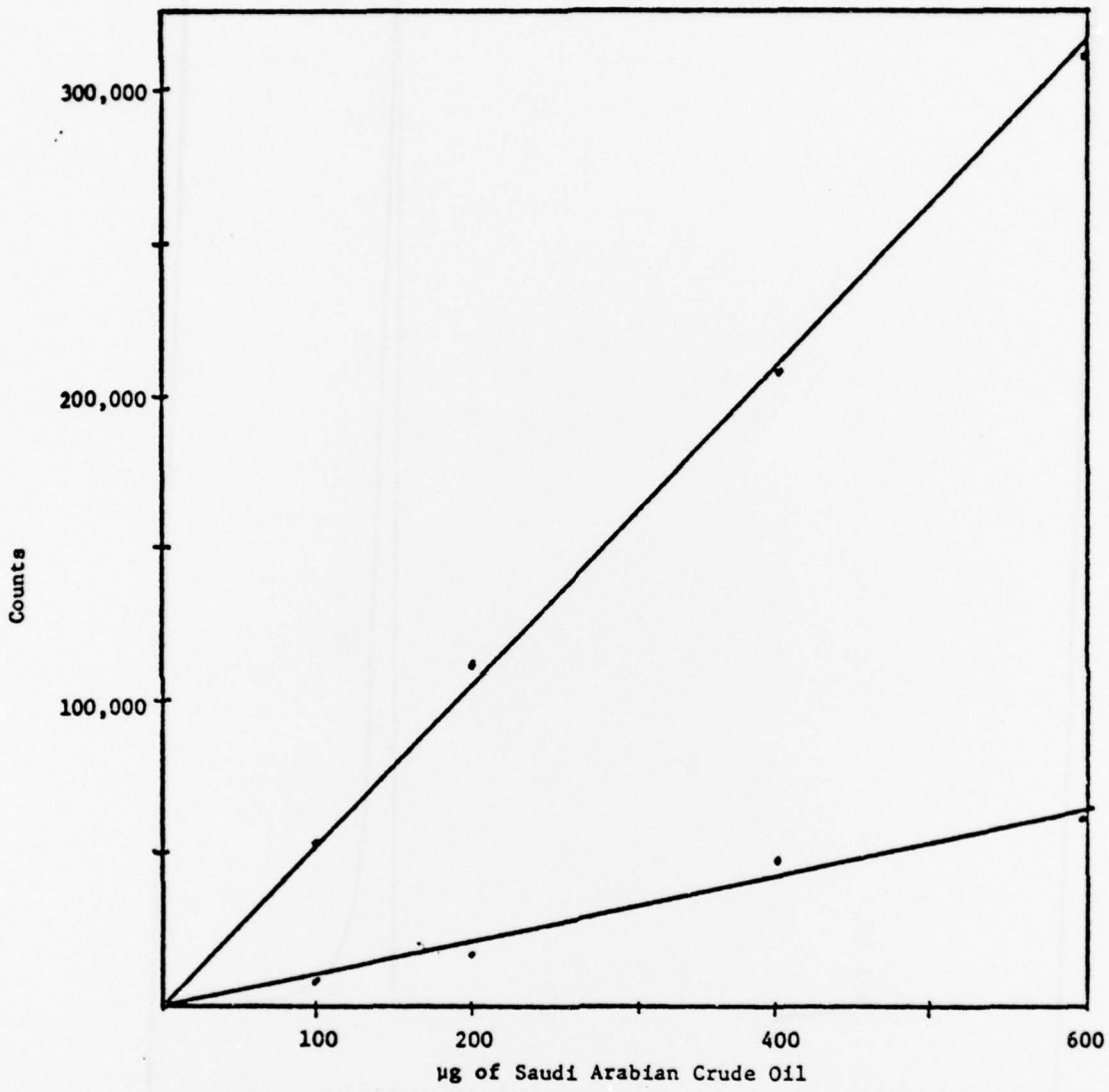


Figure 26: Calibration Graph of Saudi Arabian Crude Oil and Shrimp to Which Saudi Arabian Crude Oil Has Been Added. The Fluorescence Detector Has An Excitation Wavelength of 403 nm With a KV 418 Emission Filter.

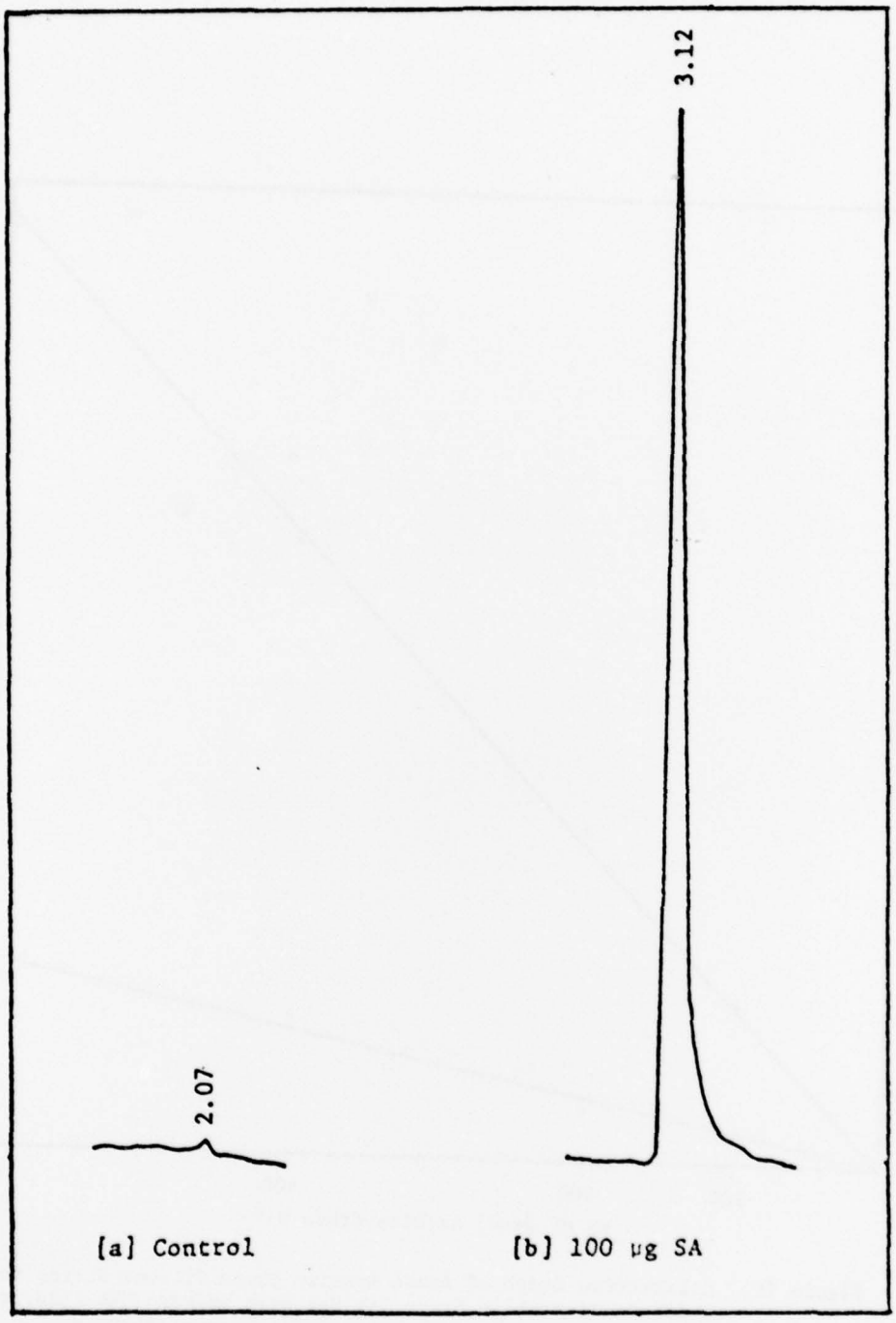


Figure 27. HPLC Chromatograms of (a) Control Sea Water and (b) Sea Water to Which 100 mg of Saudi Arabian Has Been Added. The Fluorescence Detection Excitation Wavelength Was 403 nm With a KV 418 Emission Filter. (Numbers above peaks are retention times in minutes.)

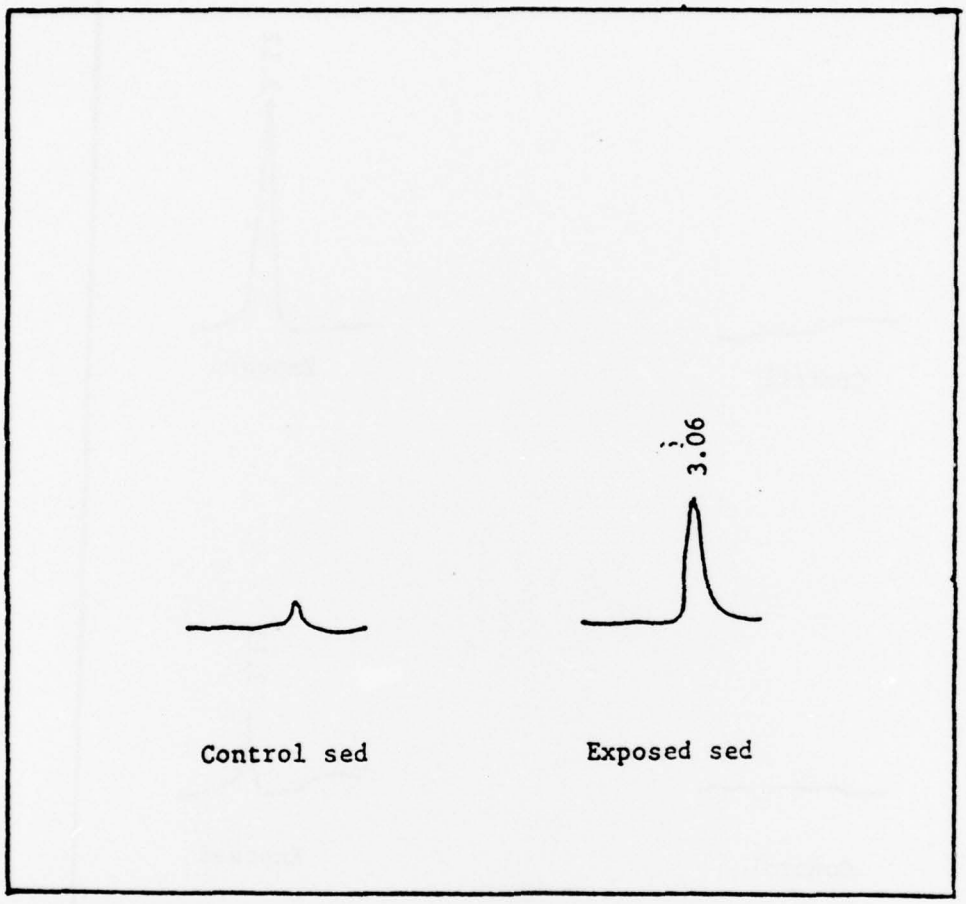


Figure 28. HPLC Chromatograms of Sediment Samples From (a) A Control Pond and (b) A Pond to Which Saudi Arabian Had Been Added 10 Months Before the Sample was Taken. The Fluorescence Detection Wavelength is 403 nm with a KV 418 Emission Filter. (Numbers above peaks are retention times in minutes.)

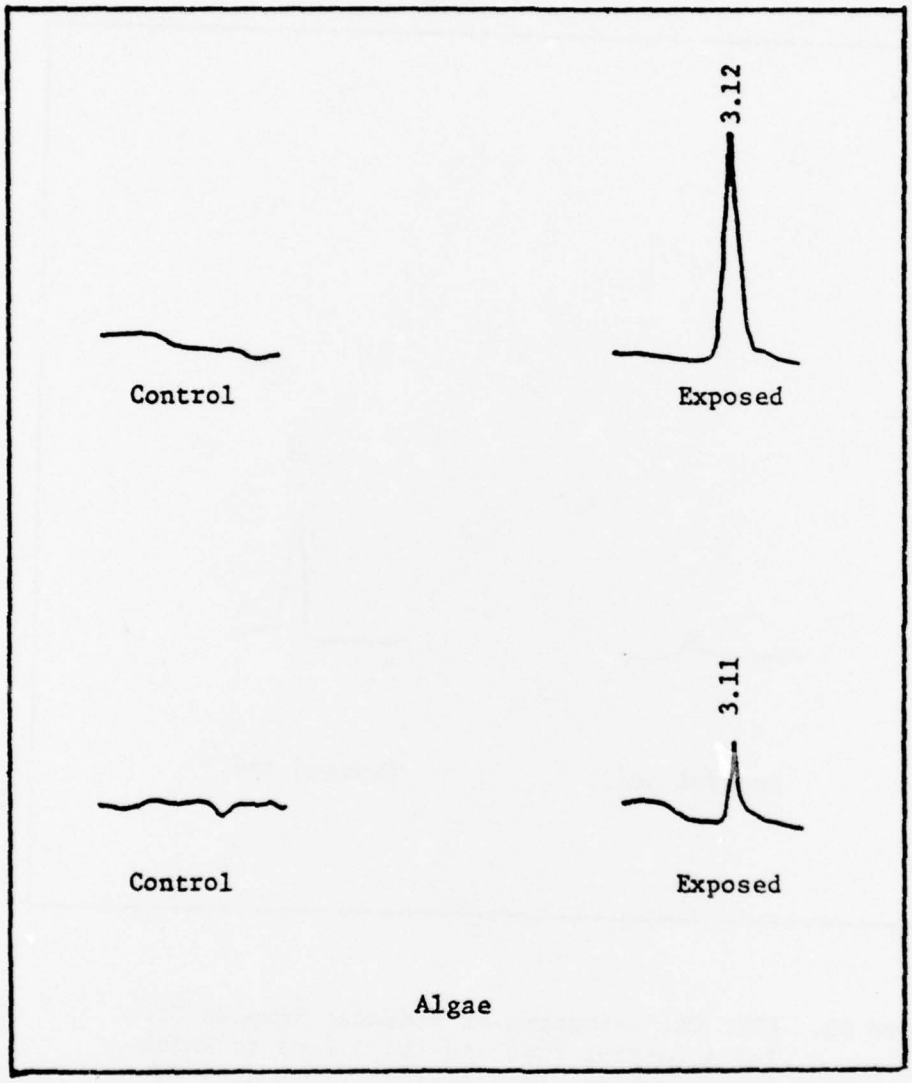


Figure 29: HPLC Chromatograms of Control Algae and Algae Grown in the Presence of 4 mg/l Saudi Arabian Crude for 96 Hours Using a Fluorescence Detector at an Excitation Wavelength of 403 nm and With a KV 418 Emission Filter. (Numbers above peaks are retention times in minutes.)

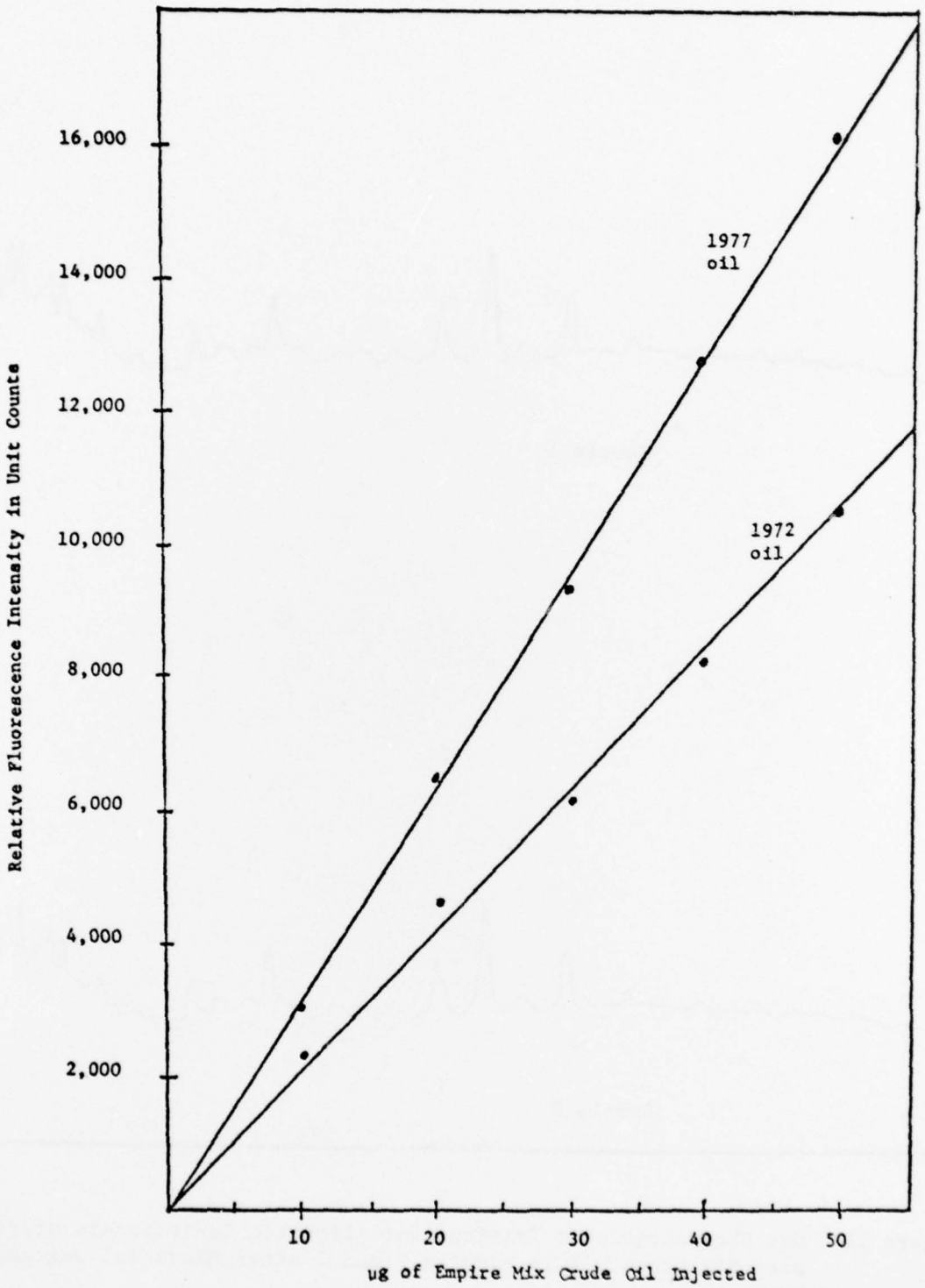


Figure 30. Relative fluorescence intensity for two samples of Empire Mix crude oil using chloroform as the solvent, excitation at 403 nm and emission at 418 nm.

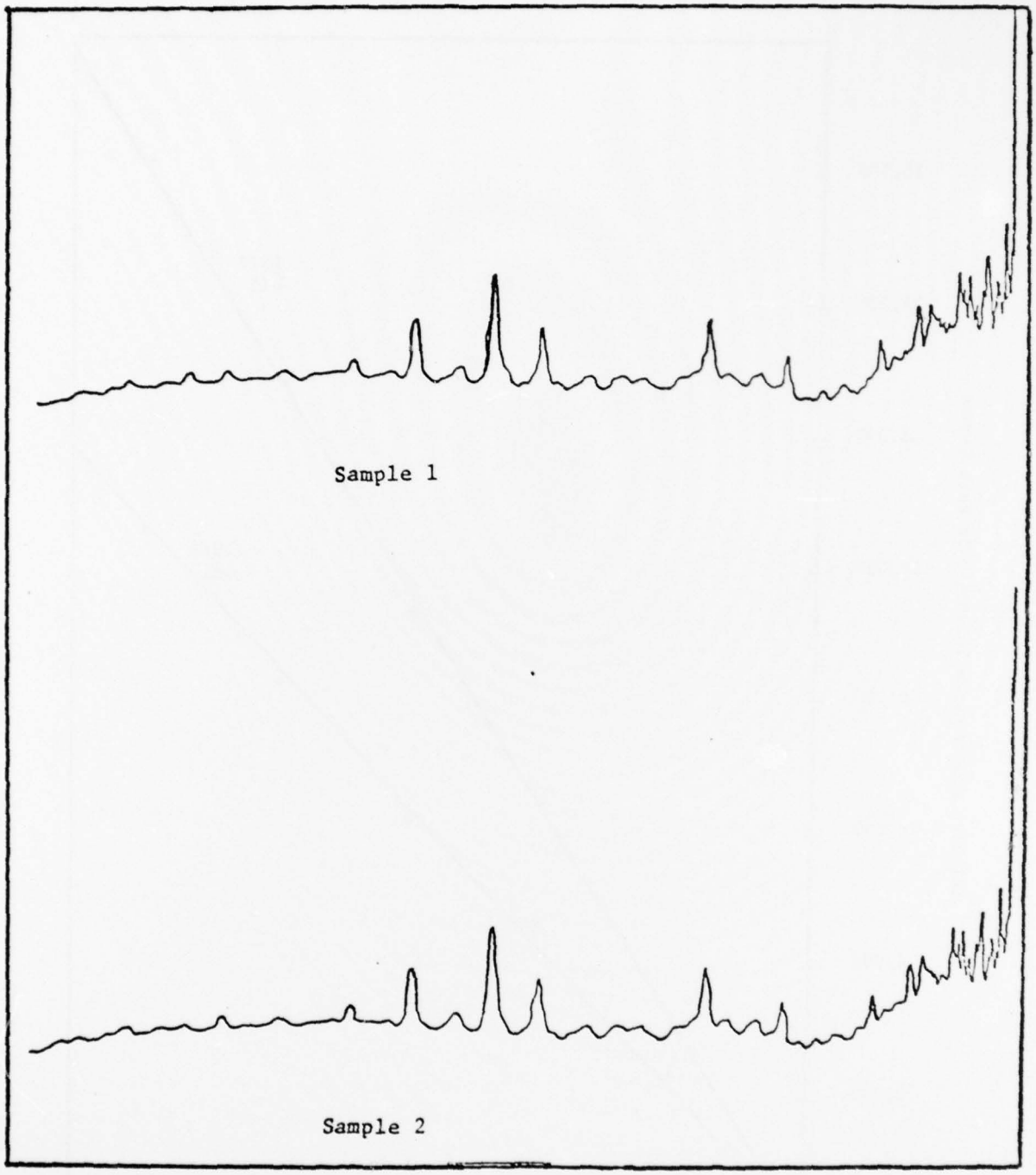


Figure 31: Gas Chromatographic Tracings for Aliphatic Constituents of Empire Mix Crude Oil in Samples 1 and 2 after Microbial Degradation.

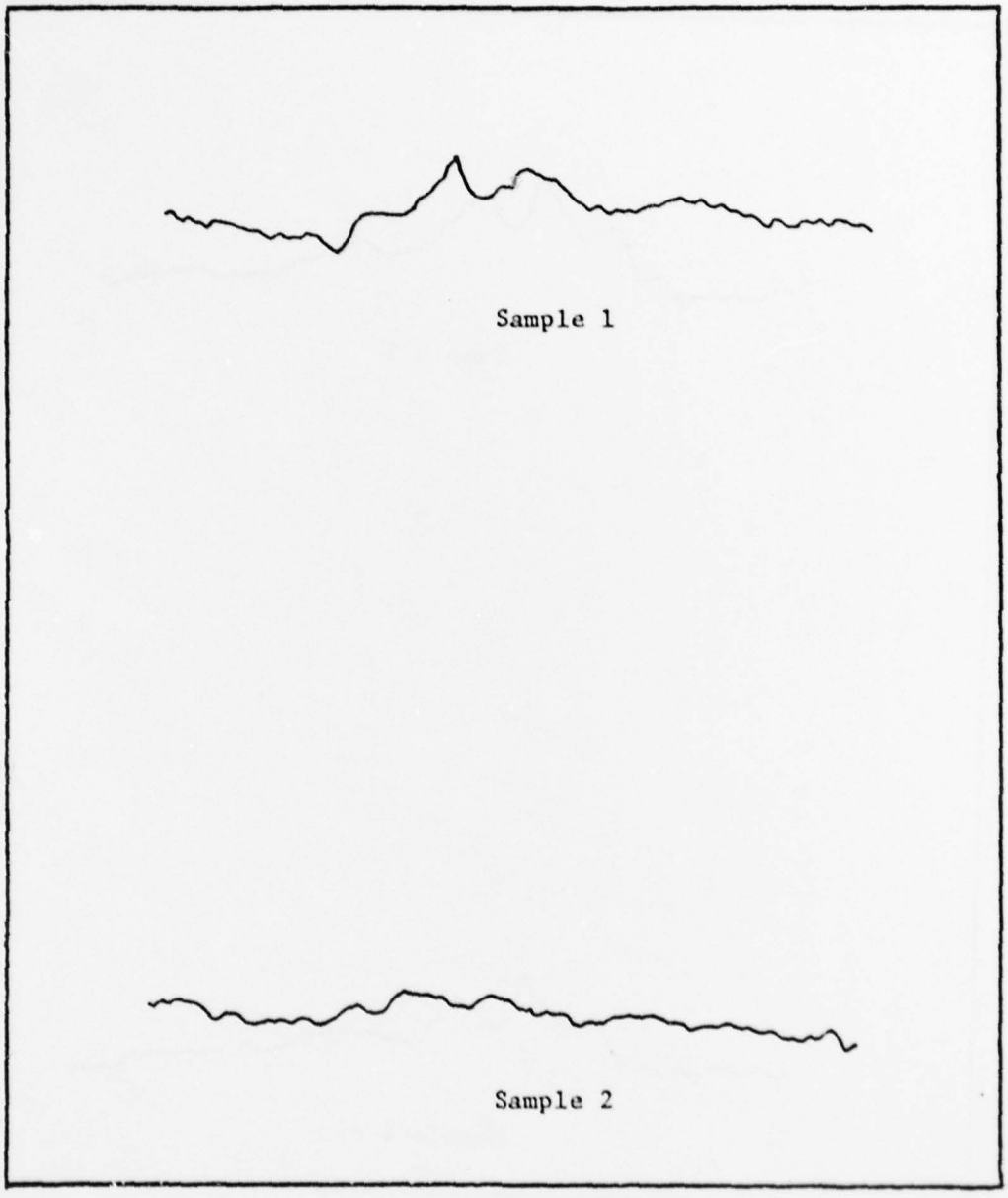


Figure 32: Liquid Chromatographic Tracing for Aromatic Constituents of Empire Mix Crude Oil in Samples 1 and 2 after Microbial Degradation Taken at 274 nm using MeOH-H_2O.

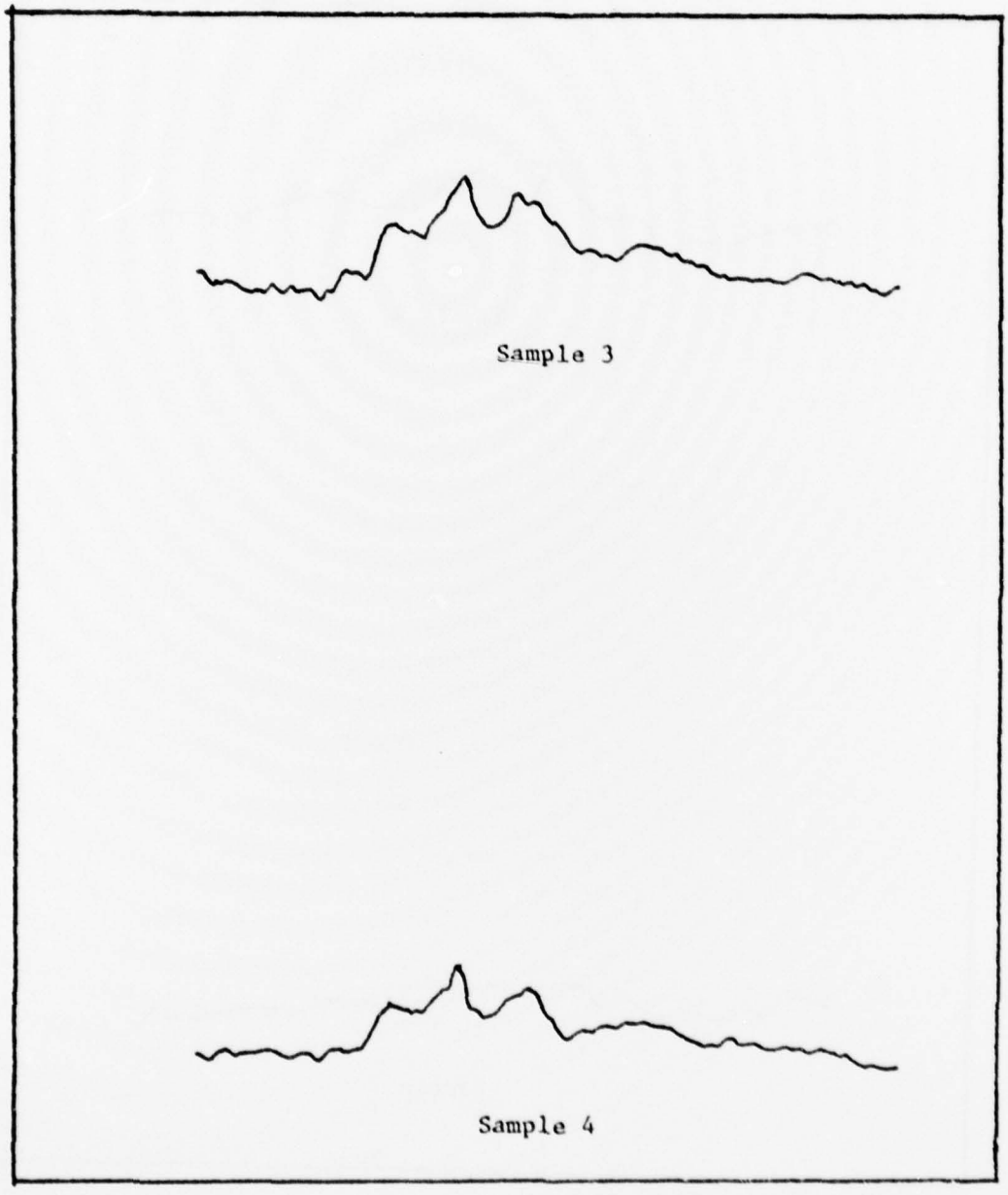


Figure 33: Liquid Chromatographic Tracing for Aromatic Constituents of Empire Mix Crude Oil in Samples 3 and 4 After Microbial Degradation Taken at 274 nm Using MeOH-H_2O.

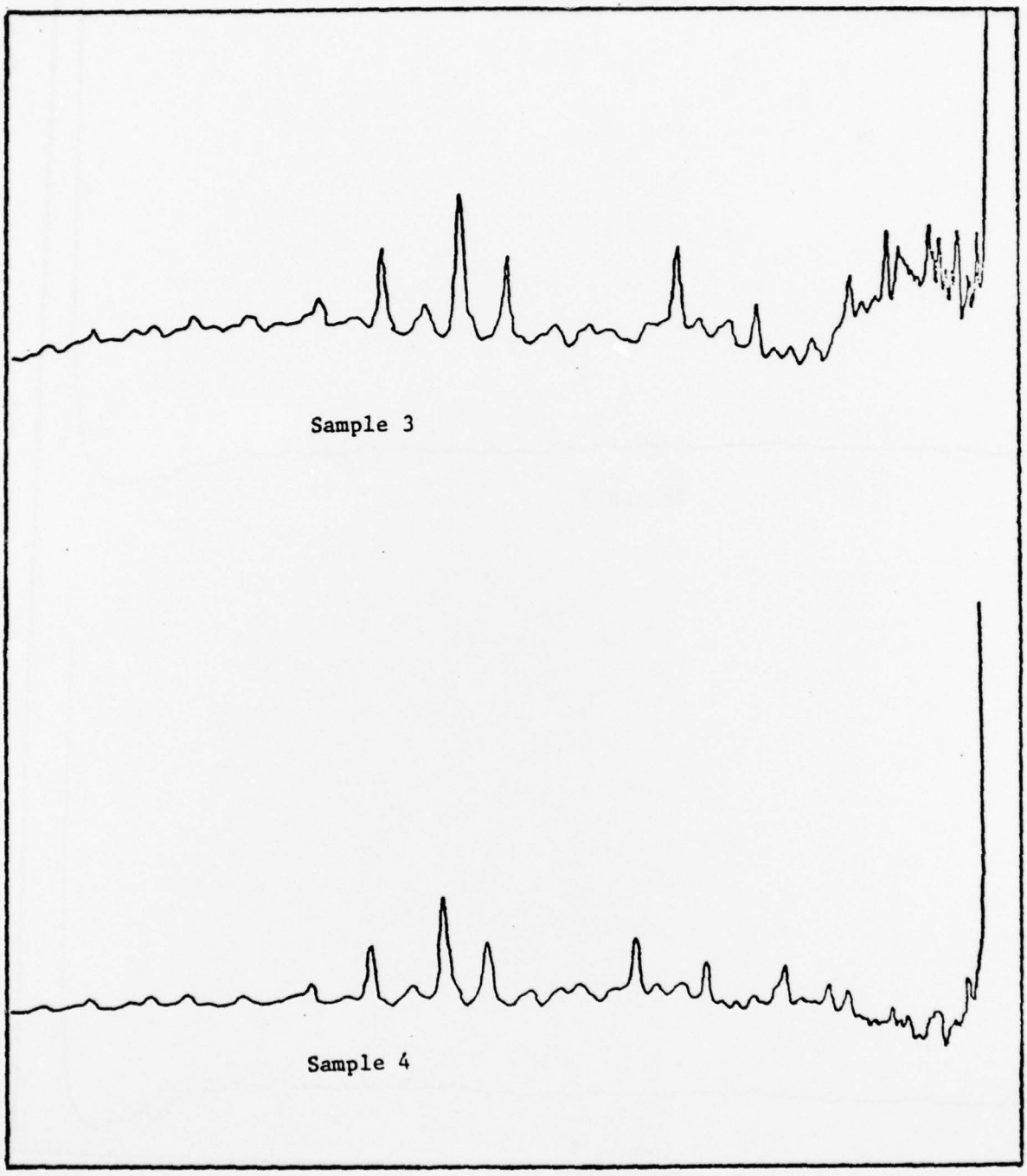


Figure 34: Gas Chromatographic Tracings for Aliphatic Constituents of Empire Mix Crude Oil in Samples 3 and 4 After Microbial Degradation.

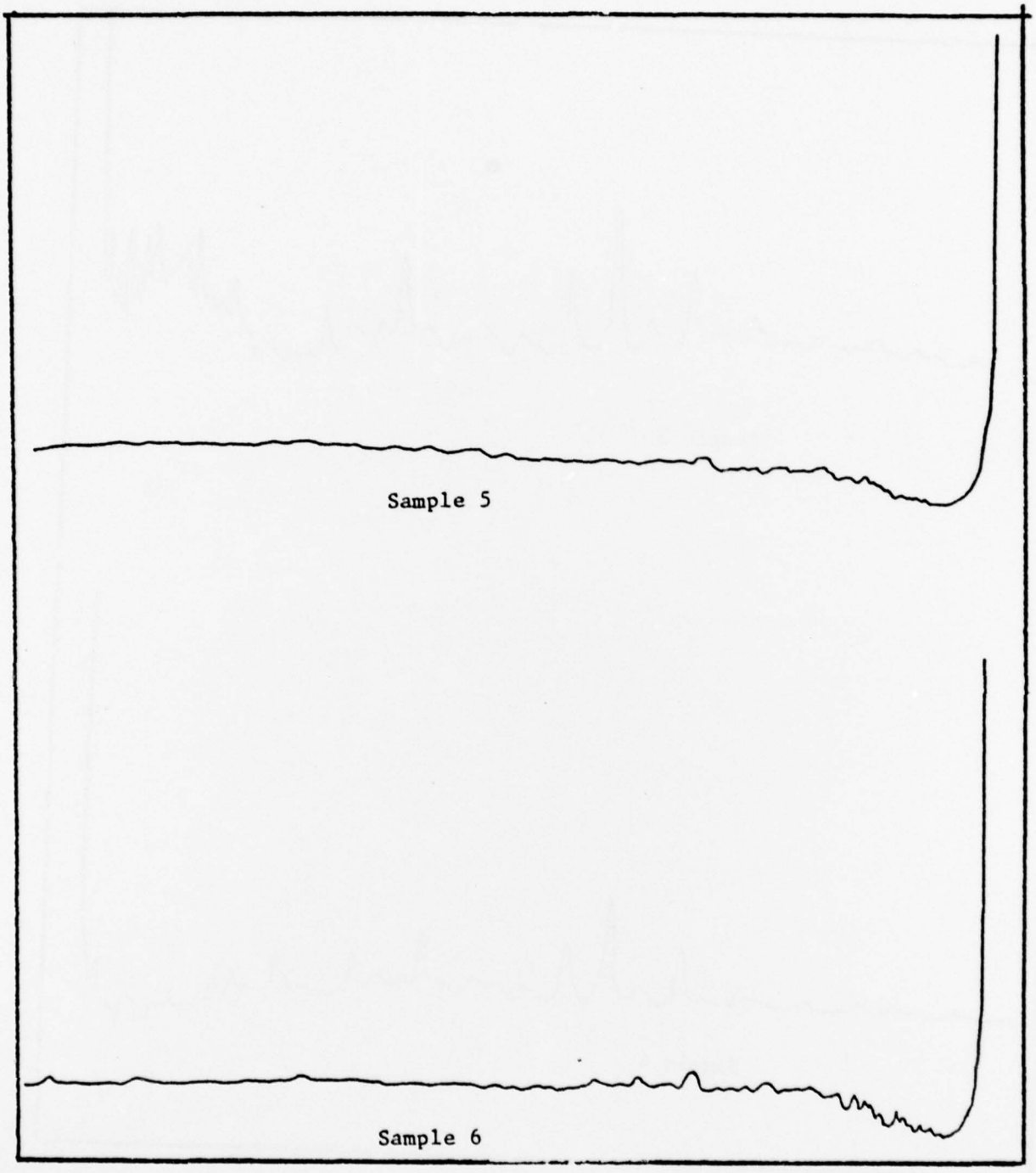


Figure 35: Gas Chromatographic Tracings for Aliphatic Constituents of Empire Mix Crude Oil in Samples 5 and 6 After Microbial Degradation.

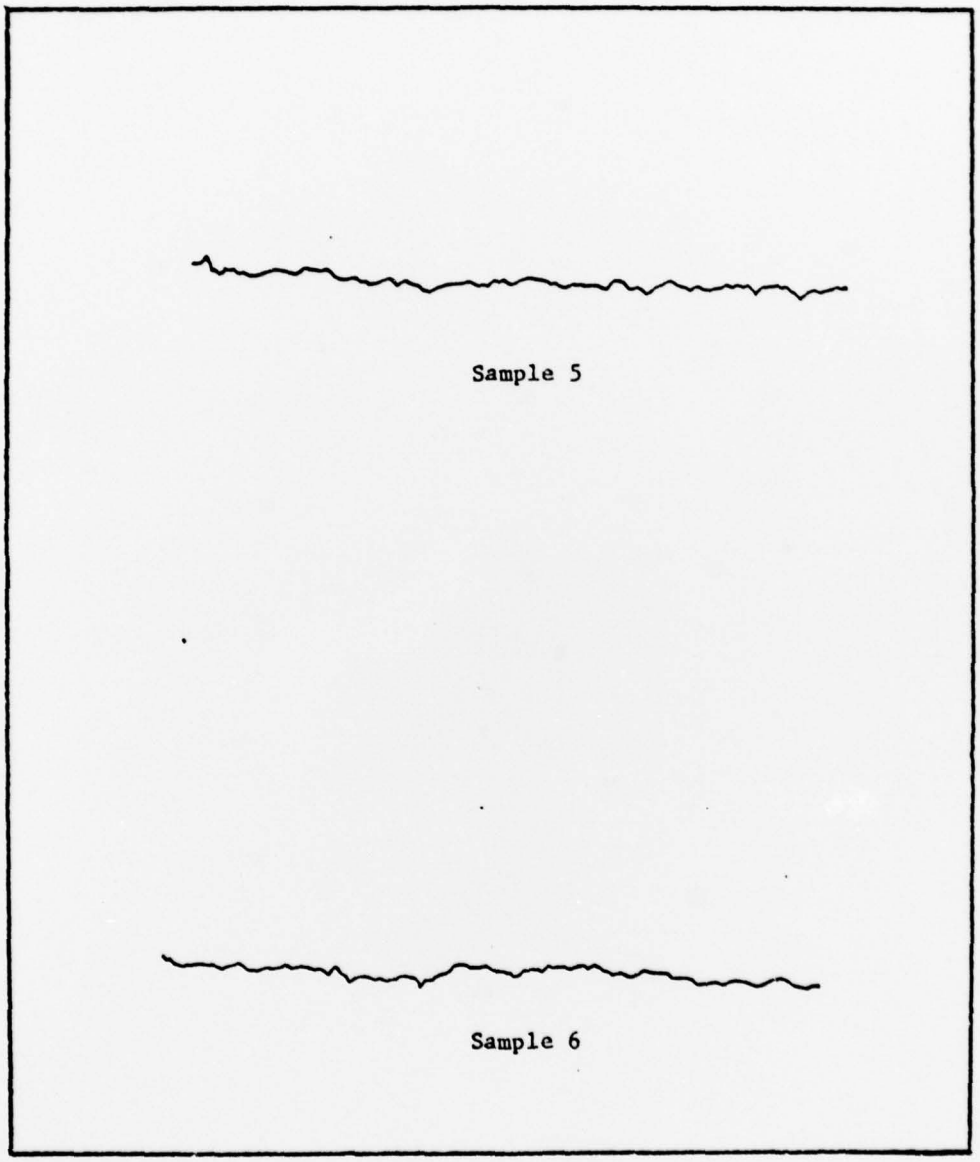


Figure 36: Liquid Chromatographic Tracing for Aromatic Constituents of Empire Mix Crude Oil in Samples 5 and 6 after Microbial Degradation Taken at 274 nm Using MeOH-H_2O.